POST-COPULATORY SEXUAL SELECTION AND THE EVOLUTION OF ADAPTATIONS TO SPERM COMPETITION RISK IN AN EXTERNALLY FERTILISING FISH

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ABSTRACT

There is extensive evidence from a wide range of taxa that supports sperm competition theory, including studies that show males can adapt to sperm competition by producing more competitive ejaculates. Several traits, including sperm concentration and velocity, influence ejaculate quality by altering male reproductive success when sperm compete. Emerging evidence shows that males can strategically alter ejaculate quality in response to cues, such as the presence of a male competitor, that signal changed sperm competition risk. However, when rapid adjustments to ejaculates occur it is unclear whether changes to sperm performance are due to changes in seminal fluid composition and/or the production of new sperm. Furthermore, if changing sperm performance is mediated by seminal fluid, the mechanisms underlying the way sperm and seminal fluid interact in most taxa are poorly understood. Here, I used a series of experiments on an externally fertilising fish, chinook salmon (*Oncorhynchus tshawytscha*), a species with a dynamic social environment in which males adopt alternative reproductive tactics that exposes them to different sperm competition risk. Overall, my aim was to determine whether male chinook salmon can make rapid strategic adjustments to ejaculate quality, and to further understand the proximate mechanisms behind such adjustments, focusing on sperm velocity - a key trait that impacts reproductive success in salmonids.

In **Chapter two**, I report results from a comprehensive series of experiments on fully grown "hooknose" males. Using a two-stage social status manipulation, I tested whether ejaculate quality traits respond rapidly to changes in sperm competition risk. I then used *in-vitro* ejaculate manipulations to determine if changes to sperm velocity are mediated by seminal fluid. Finally, using *in-vitro* fertilisation trials in which sperm compete under simulated natural spawning conditions, I assessed the paternity share of males using both manipulated and unmanipulated ejaculates. I found that both sperm number and velocity were linked to social position; subdominant males that have greater sperm competition risk produced higher quality ejaculates compared to dominant males. Furthermore, males changing from dominant to subdominant status responded to this increase in sperm competition risk by producing ejaculates with faster swimming sperm within 48 hours. By manipulating ejaculates, I found that rapid changes in sperm velocity were mediated by seminal fluid and found that the effect

of seminal fluid on sperm velocity directly impacted paternity share and therefore reproductive success.

Chapter two provides compelling support for seminal fluid having "quality" driven effects that influence sperm from all males similarly rather than a "targeted" negative effect on rival male sperm. However, two studies have reported such targeted effects in externally fertilising fish, but both compared averages across treatment groups. In **Chapter three**, I test the hypothesis that support for quality driven effects is only apparent when relative sperm velocity between males in each pair is considered. I tested this hypothesis using an experiment that manipulated ejaculates from male chinook salmon with different life-histories, "hooknose" males and early maturing "precocious" males. Although comparison of averages across treatment groups suggested targeted effects on rival sperm, the alternate approach found a significant correlation between relative sperm velocity and changes in sperm velocity caused by rival seminal fluid. These results further support a quality driven rather than targeted effect and provide further information about the possible proximate mechanism of sperm and seminal fluid interaction.

To gain further insight on the potential mechanism of sperm and seminal fluid interaction identified in chapters two and three, I used proteomic methods to characterise the seminal fluid proteome in chinook salmon. Growing evidence for invertebrate species shows that seminal fluid proteins (SFPs) have evolved key functional roles in sperm competition. However, relatively little is known about SFPs in vertebrate species. Chapters four and five explore the chinook salmon seminal fluid proteome, using seminal fluid collected during the social status manipulation experiment in chapter two and a combination of pre-fractionation techniques followed with analysis by mass spectrometry. In **Chapter four**, the chinook salmon seminal fluid proteome is described and compared to the three previous proteomic studies in teleost fish. To identify candidate SFPs that may be linked to the underlying mechanism, in **Chapter five** I assess the correlation between SFP abundance, male social status and ejaculate quality traits. I show that SFP composition is influenced by social status, and identify several SPFs correlated with sperm velocity and sperm concentration that are part of energy metabolism, defence and signalling pathways previously shown to influence sperm function.

In summary, using a series of behavioural manipulation, ejaculate manipulation*, in vitro* sperm competition experiments and proteomic analyses on chinook salmon males with

alternative reproductive tactics, I provide unequivocal evidence that sperm competition risk drives patterns of investment in ejaculate quality. Furthermore, I show that adjustment of sperm velocity, a key trait determining ejaculate competitiveness and impacting male reproductive success in salmonids, occurs via investment in seminal fluid. My results provide support that males invest in high quality seminal fluid that affects the velocity of sperm from all males similarly, rather than targeting and reducing the velocity of sperm from rival males. I also provide a detailed analysis of the chinook salmon seminal fluid proteome and identify a candidate list of SFPs associated with ejaculate quality, that will be critical for the identification of proximate mechanisms underlying sperm and seminal fluid interactions that influence male reproductive success. These combined results represent a significant advance in our understanding of post-copulatory sexual selection and the evolution of adaptations to sperm competition risk.

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TABLE OF CONTENTS

LIST OF FIGURES PAGE

LIST OF TABLES PAGE

CHAPTER ONE
INTRODUCTION

1.1 GENERAL INTRODUCTION AND THESIS LAYOUT

The content presented in this thesis is formatted to include one manuscript published in *eLife* (Chapter Two), one manuscript that is nearly ready for submission to *Biology Letters* (Chapter Three), and one manuscript that is in preparation for submission to a Special Issue entitled, "Reproductive Proteomics Comes of Age", in *Molecular & Cellular Proteomics* (Chapters Four and Five). Given the manuscript style of this thesis, each data chapter consists of an abstract, introduction, materials and methods, results, discussion and references sections. For the purpose of thesis cohesion and clarity between chapters, each data chapter begins with a preface. In each preface, a brief outline of the purpose of the data chapter and any additional information not included in the manuscript is provided.

This thesis includes work from a range of biological disciplines including behavioural ecology, sperm biology and proteomics. Collaborative work was therefore required from researchers specialising in different areas to fulfil the requirements of each study. The roles of co-authors who assisted in the research presented in each chapter are listed after the title, others are mentioned in the acknowledgements section of each manuscript.

This introduction chapter is divided into six main sections including this one. In Sections 1.2-1.5 I provide the necessary theoretical background and develop the context and rationale for the research conducted in this thesis. In Section 1.2 I begin with an overview of postcopulatory sexual selection and sperm competition theory. I then follow this with a discussion of traits that influence the competitive performance of ejaculates (i.e. "ejaculate quality") in Section 1.3. In Section 1.4, I review the evidence that males from a range of species can make rapid adjustment to ejaculate quality and present case studies that highlight the role of seminal fluid and seminal fluid proteins and their impact on ejaculate quality and competitiveness. In Section 1.5, I provide an overview of the reproductive biology of my study species, the Chinook salmon (*Oncorhynchus tshawytscha*), reviewing what we currently know about ejaculate investment strategies and the roles seminal fluid play in salmonid

reproductive biology. Lastly, I outline the general aims and scope in the summary section of this chapter (section 1.6).

1.2 POSTCOPULATORY SEXUAL SELECTION AND SPERM COMPETITION RISK

Sexual selection is the evolutionary mechanism that results in an increase in the frequency of alleles bestowing a reproductive advantage (Birkhead and Pizzari 2002). Darwin (1871) described cases of sexual selection in animals where many examples are accredited to male-male competition for mates and female mating preferences. Competition among males for mating opportunities provides a strong selective force shaping the evolution of elaborate traits across a wide range of life and when females mate with multiple males, this potent evolutionary force continues to act post-mating, resulting in competition among sperm as they attempt to fertilise that female's ova (Birkhead and Pizzari 2002, Andersson and Simmons 2006).

When Parker (1970) determined that selection can continue after copulation has occurred, he made one of the major advances in sexual selection theory (Andersson and Simmons 2006). Postcopulatory sexual selection occurs when females mate promiscuously, which is now recognised as widespread across the animal kingdom (Birkhead and Møller 1998, Birkhead and Pizzari 2002, Zeh and Zeh 2003, Simmons 2005, Parker and Birkhead 2013, Taylor et al. 2014), allowing competition between gametes to occur right up until fertilisation occurs (Birkhead and Pizzari 2002, Andersson and Simmons 2006). "Sperm competition" is defined as competition between sperm from different males to fertilise a given set of a female's ova (Parker 1970). A female's ability to influence the outcome of sperm competition by altering sperm performance and thus bias paternity success towards a preferred male is termed "cryptic female choice" (Eberhard and Cordero 1995). Both forms of postcopulatory sexual selection create powerful selective forces that shape the evolution of reproductive traits. For example, the large variation in male genital morphology between closely related species is now known to be a consequence of postcopulatory sexual selection, as variation in genital traits directly influences reproductive success (Birkhead and Møller 1998, House and Simmons 2003, Hosken and Stockley 2004, Andersson and Simmons 2006). In addition, sperm competition theory predicts that species experiencing a greater risk of sperm competition produce ejaculates containing more sperm (Parker 1990b). This is consistent with the discovery that many species have large testis relative to body size (GSI) that experience high

levels of sperm competition, compared to those with low sperm competition risk (Stockley et al. 1997, Birkhead and Møller 1998, Byrne et al. 2002, Birkhead and Pizzari 2002, Ramm et al. 2014, Parker 2016).

Due to sperm competition favouring larger ejaculates with greater sperm numbers, it is important to understand how males maximise their reproductive success via strategic investment in sperm production and allocation under competitive conditions (Parker and Pizzari 2010). A series of models termed "sperm competition games" have been developed by Parker and colleagues (Parker 1990a, 1990b, 1993, Parker and Begon 1993, Parker et al. 1996, 1997, Ball and Parker 2000, Parker et al. 2013) that attempt to predict the evolutionary stable strategy for male investment in ejaculates under various sperm competition scenarios (reviewed by Parker 1998; Parker & Pizzari 2010). All models assume that males have a fixed energy budget for reproduction, which can be allocated either to ejaculate expenditure (factors influencing ejaculate quality discussed below) or mating expenditure (searching for and fighting over mates), creating a trade-off between these components (Parker 1998; Parker & Pizzari 2010). Many models operate as a form of raffle, where increasing sperm numbers increases a male's chance of fertilising a female's eggs when the raffle is "fair" (Parker 1990b). "Loaded" raffles devalue the sperm of one male relative to his competitor based upon occupation of either favoured or disfavoured mating roles, such as mating first or second (Parker 1990b). The information about the level of sperm competition risk available to each male, for instance in "sneak-guard" mating systems where guard males are unaware of the presence of sneaks, also influences the predicted strategies for that male (Parker 1990b).

We can make several predictions about male investment in ejaculate expenditure based on these models (Parker 1998, Parker and Pizzari 2010):

1. When sperm competition risk is low, virtually all reproductive energy will be spent on mating expenditure. As sperm competition risk increases males should allocate more energy into ejaculate expenditure.
2. When a male has a fixed role so that he is always disfavoured, we expect that male to compensate by increasing allocation to ejaculate expenditure.
3. When a male's sperm competition risk changes, that male should adjust his ejaculate expenditure accordingly in order to maximise reproductive success.

4. When a male has more information about the risk of sperm competition than his competitor, that male should spend more on ejaculate expenditure. The disparity in expenditure between these males should increase as general sperm competition risk decreases.
5. In species where many males typically engage in sperm competition, termed sperm competition "intensity" rather than risk, investment in ejaculates is predicted to decrease when the number of competitors increases above two. This is because as the number of males increases above two the benefit of per unit expenditure on ejaculates decreases.

This provides a basis for making explicit hypotheses about the strategic investment in ejaculate expenditure by males in a number of scenarios. The following section will review the ways in which males can alter ejaculate expenditure to increase their chances of fertilisation success when in competition with a rival's sperm.

1.3 FACTORS CONTRIBUTING TO EJACULATE QUALITY

As discussed above, producing ejaculates containing more sperm is one way to increase the chance of fertilisation success under sperm competition conditions, consistent with the increase in relative testis size found in species with greater sperm competition risk (Birkhead and Møller 1998, Byrne et al. 2002, Birkhead and Pizzari 2002, Ramm et al. 2014). Since sperm competition selects for greater sperm production, we can also expect adaptations that increase the efficiency of spermatogenesis within the testis which may differ from simply increasing testis size (Lüpold et al. 2009b, 2011, Ramm and Schärer 2014, Ramm et al. 2014). For example, New World blackbird (Icteridae) species with greater levels of sperm competition have increased proportions of tissue within the testes involved in spermatogenesis (Lüpold et al. 2009b, 2011). Producing greater numbers of sperm, however, is not the only adaptation that increases a male's chance of fertilisation success when sperm compete (Snook 2005). "Sperm quality" is the term used to describe any combination of sperm traits that influence male fertility (Snook 2005, Fitzpatrick and Lüpold 2014). Sperm characteristics that are likely to influence their competitive ability include sperm morphology, longevity, viability, mobility and velocity (Snook 2005, Fitzpatrick and Lüpold 2014).

1.3.1 *Sperm morphology and velocity*

Sperm morphology is highly variable among species and the production of varied sperm forms is likely linked to sperm competition (Snook 2005, Ramm and Schärer 2014, Fitzpatrick and Lüpold 2014, Ramm et al. 2014). Sperm flagella length is predicted to increase sperm velocity, and faster sperm are predicted to be more competitive as they should be able to reach the egg more quickly (Snook 2005). However, experimental evidence for a link between sperm length and speed is mixed (Snook 2005, Fitzpatrick and Lüpold 2014). There are several studies showing that sperm velocity is positively correlated with increasing sperm length when compared among different species (Gomendio & Roldan 2008; Fitzpatrick et al. 2009; Lüpold et al. 2009a; Tourmente et al. 2009, 2011 but see Gage & Freckleton 2003). Conversely, many studies within species have found no correlation between sperm length and speed (reviewed by Humphries et al. 2008; Simmons & Fitzpatrick 2012 but see Malo et al. 2006; Fitzpatrick et al. 2010), for example longer sperm did not swim faster than shorter sperm in *Salmo salar* (Gage et al. 2002) and in *Drosophila melanogaster* larger sperm were slower than smaller sperm (Lüpold et al. 2012).

In contrast, the relationship between sperm velocity and fertilisation success is well established. Increased sperm velocity is correlated with higher fertilisation success in a range of species (Snook 2005, Simmons and Fitzpatrick 2012, Fitzpatrick and Lüpold 2014). For example, males with faster sperm had greater fertilisation success in red deer (Malo et al. 2005), fishes (Lahnsteiner et al. 1998, Jobling et al. 2002, Casselman et al. 2006) and marine invertebrates (Levitan 2000, Kupriyanova and Havenhand 2002). Studies have also shown that males with faster swimming sperm sire a greater proportion of offspring when in competition with a rival male in birds (Birkhead et al. 1999, Donoghue et al. 1999) and in many fishes (Gage et al. 2004, Burness et al. 2004, Rudolfsen et al. 2008, Ottesen et al. 2009, Gasparini et al. 2010, Boschetto et al. 2010, Evans et al. 2013, Egeland et al. 2015, Rosengrave et al. 2016). Fitzpatrick et al. (2009) found that sperm swimming speed was greater in species of cichlid fishes that experience greater levels of sperm competition. However, faster sperm may only be an advantage when fertilisation occurs as a "race to the egg" type scenario (Fitzpatrick and Lüpold 2014), and sperm longevity may be more important than velocity when females store sperm (Smith 2012). For example, larger and slower sperm are more competitive in *D. melanogaster,* where sperm that are similar in length to the female receptacle are better at

displacing and resisting displacement of smaller rival sperm (Lüpold et al. 2012). Fitzpatrick et al. (2012) found that in the mussel *Mytilus galloprovincialis* slower swimming and longer-lived sperm were advantageous when gametes were released in low densities, possibly because they have longer to search for eggs.

1.3.2 *Ejaculate quality and seminal fluid proteins*

Ejaculates contain not only sperm but also a non-sperm component, typically referred to as "seminal fluid", that can contain proteins, immunopeptides and a range of metabolites (Poiani 2006, Perry et al. 2013). Seminal fluid can have a considerable influence on fertilisation and should be studied in more detail to understand ejaculate quality rather than focusing on sperm traits solely as main divers of male fertility and fitness (Poiani 2006, Cameron et al. 2007, Perry et al. 2013, McGraw et al. 2014). Several seminal fluid constituents, in particular Seminal Fluid Proteins (SFPs), have important effects on sperm, influencing sperm survival and fertilisation ability (Eberhard and Cordero 1995, Glander et al. 1996, Henricks et al. 1998, den Boer et al. 2008b, Holman 2009, den Boer et al. 2010b, King et al. 2011, Rodríguez-Martínez et al. 2011, Simmons and Beveridge 2011, Mendoza et al. 2013, Rodrigues et al. 2013). Other SFPs can have important influences on female behaviour and physiology that influence the reproductive fitness of both sexes (Chapman and Davies 2004, Robertson 2005, Chapman 2008, Avila et al. 2011, Schjenken and Robertson 2014, Sirot et al. 2015).

Reproductive proteins can regulate essential processes involved in fertilisation and therefore have a direct influence on male and female fitness (Clark et al. 2006). Elevated substitution rates and a high degree of polymorphism generally characterise the molecular evolution of reproductive proteins (Swanson and Vacquier 2002, Andrés et al. 2006, Clark et al. 2006, Karn et al. 2008, Ramm et al. 2009). SFP composition can be highly variable among species; for example, Druart et al. (2013) compared the seminal fluid proteome of seven domestic mammalian species and found that the percentage of proteome common between species ranged from 73% between sheep and goat to only 4% between goat and alpaca. The degree of similarity between these species seminal fluid proteomes is correlated with how closely related they are and may also reflect differences in mating systems (Druart et al. 2013). It's suggested that sperm competition and coevolution between male and female proteins in the context of sexual selection and/or sexual conflict are selective drivers of reproductive protein diversification (Swanson and Vacquier 2002, Clark et al. 2006, Wong et al. 2011). For example,

comparisons among primates have found that polyandrous species have greater rates of evolution for the SEMG2 gene, which encodes for the main structural protein in semen coagulum, involved in the formation of a copulatory plug (Dorus et al. 2004, Ramm et al. 2007). Additionally, Clark & Swanson (2005) found that loss of function at the TGM4 gene, which codes for a protein needed for the formation of semen coagulum, has occurred in gorillas and gibbons, both of which are monandrous species.

A great deal of research on SFPs has been conducted in insects, with proteins identified that are predicted to function in several processes including sperm maintenance and storage, direct influences on female gene expression, physiology and behaviour, as well as immunity-related functions (Avila et al. 2011). The role of SFPs in reproduction has been extensively researched in *D. melanogaster,* with 133 proteins identified as potentially transferred to females during copulation (Chapman 2008). Natural variation in genes coding for male reproductive proteins is linked to reproductive success in *D. melanogaster*, with six out of the ten genes studied showing significant associations with sperm competition phenotypes (Fiumera et al. 2004). Some of these proteins have functions that can influence sperm competition, for instance the accessory gland protein, Acp36DE, is required for sperm storage within the female reproductive tract and this is key for male reproductive success (Neubaum and Wolfner 1999, Tram and Wolfner 1999, Chapman et al. 2000, Bloch Qazi and Wolfner 2003, Avila and Wolfner 2017). Another accessory gland protein Acp70A or "sex peptide" has been studied extensively and elicits a range of postmating effects in females (Chapman 2001, 2008, Wolfner 2002, Chapman and Davies 2004, Avila et al. 2011). Sex peptide physically binds to sperm for delivery into female sperm storage, where it is gradually cleaved from sperm, facilitating long term effects that requires multiple SFPs working in conjunction (Peng et al. 2005a, Ravi Ram and Wolfner 2007a, 2007b, 2009). This results in multiple behavioural and physiological effects on females, including stimulation of egg laying (Ravi Ram and Wolfner 2007a), reduced female receptivity to remating (Chapman et al. 2003, Liu and Kubli 2003, Ravi Ram and Wolfner 2009), and altered feeding (Carvalho et al. 2006), sleeping (Isaac et al. 2010) and aggressive (Bath et al. 2017) behaviours. These responses are accomplished by interaction with neurons (Häsemeyer et al. 2009, Yang et al. 2009, Kubli and Bopp 2012, Rezával et al. 2012) and results in changes in the expression of many genes (McGraw et al. 2004, Peng et al. 2005b, Domanitskaya et al. 2007).

Several examples illustrate the potential for seminal fluid, particularly SFPs, to influence male fertilisation success and therefore the outcome of sperm competition by directly affecting sperm quality traits. Social Hymenoptera (ants and bees) produce SFPs that enhance sperm viability (den Boer et al. 2008a, 2009, King et al. 2011) and in polyandrous species, while SFPs have positive effects on own sperm viability, they are implicated in the incapacitation of rival male's sperm (den Boer et al. 2010a, 2015). In humans, the SFP α_2-macroglobin was correlated with both the percentage of motile sperm and sperm velocity (Glander et al. 1996) and Insulin-like growth factor is associated with normal sperm development (Glander et al. 1996). Selenoprotein-P concentration in human seminal fluid was correlated with sperm count and the percentage of vital sperm (Michaelis et al. 2014). Rodrigues et al. (2013) found differential expression of the seminal fluid proteome in Santa Ines rams between ejaculates containing > or < 80% motile sperm, with arylsulfatase A and zinc-alpha-2-glycoprotein associated with greater sperm motility. These examples suggest that studies of sperm competition should consider both sperm and seminal fluid in order to assess male investment in ejaculate expenditure. In the next section I review the evidence for differential investment in ejaculate expenditure by males in different mating roles.

1.4 MATING ROLES AND EJACULATE ALLOCATION STRATEGIES

In addition to differences among species in ejaculate composition we also expect that intraspecific variation in sperm competition risk should result in adaptive changes in ejaculate expenditure (Parker and Pizzari 2010, Perry et al. 2013, Fitzpatrick and Lüpold 2014). Often species have mating systems where males occupy either favoured or disfavoured mating roles, these roles are usually associated with copulation order, and sperm from males occupying favoured roles more likely to fertilise a female's ova (Parker 1998, Parker and Pizzari 2010). Males can occupy roles randomly (Parker 1998, Parker and Pizzari 2010); for example, in *D. melanogaster*, the male in the favoured mating role is the 2nd to mate with a female (Price 1997, Snook and Hosken 2004, Manier et al. 2010), therefore males do not occupy fixed roles but are assigned roles at the time of copulation depending on female mating status (virgin or already mated). Roles can also be fixed and associated with male phenotype (Parker 1998, Parker and Pizzari 2010). For example, many species have males that display alternative reproductive tactics (ARTs), with fully grown "guard" males occupy a favoured mating role by monopolising mating opportunities, and males that mature

precociously and attempt to "sneak" fertilisations with females (Gross 1996, Taborsky 1998). We expect that when a male has a fixed role so that he is always disfavoured, that male will compensate by increasing allocation to ejaculate expenditure (Parker 1998, Parker and Pizzari 2010). Since males adopting a sneaking tactic will almost always experience sperm competition with guards (and therefore have more information about the level of sperm competition) we expect these males to invest more in ejaculate quality than guard males (Parker 1990a, Parker and Pizzari 2010). Males with a guarding strategy will additionally trade-off between ejaculate and mating expenditure, as they expend energy securing territory or finding mates (Parker 1990a, Parker and Pizzari 2010). For example, in several fish species with males that adopt ARTs based on age of sexual maturity, sneaker males produce higher quality ejaculates with either more sperm (Vladić and Jarvi 2001, Vladić et al. 2002, Liley et al. 2002, Neff et al. 2003) or faster swimming sperm (Burness et al. 2004; Fitzpatrick et al. 2007; Locatello et al. 2007; Evans 2010; Smith & Ryan 2010; Flannery et al. 2013 but see Burness et al. 2005) and sneakers were found to have greater or equal fertilisation success in Salmonids (Vladić et al. 2002, 2010, Young et al. 2013).

1.4.1 *The social environment and plasticity in ejaculate expenditure*

Given that expenditure for reproduction is limited and ejaculate production is costly (Dewsbury 1982, Olsson et al. 1997, Sirot et al. 2009, Perry and Tse 2013), males are confronted with trade-offs between ejaculate investment per copulation and total investment in ejaculates over a lifetime, resulting in differential investments among males to maximise reproductive success (Parker 1998). Therefore, a key component of male reproductive fitness is the ability to adjust ejaculate expenditure in response to changing social cues and sperm competition risk (Wedell et al. 2002, Bretman et al. 2011a). Several studies have found that males can indeed adjust ejaculate expenditure in response to the presence of rival males (Zbinden et al. 2003, 2004, Kilgallon and Simmons 2005, Bretman et al. 2009, 2010, 2012, Smith and Ryan 2011, Moatt et al. 2014, Fitzpatrick and Lüpold 2014, Burger et al. 2015b) and this is supported by meta-analyses demonstrating that across a wide range of taxa, males transfer larger ejaculates to females when exposed to a single rival (delBarco-Trillo 2011, Kelly and Jennions 2011).

Other studies have found that males can adjust ejaculate expenditure if they change mating role, such as changing from subdominant to dominant social status (Rudolfsen et al. 2006,

Pizzari et al. 2007, Cornwallis and Birkhead 2007, Fitzpatrick et al. 2008, Kustan et al. 2011). Males can additionally adjust ejaculate expenditure in response to perceived female quality, compatibility or mating status (Cornwallis and Birkhead 2006, Gasparini et al. 2009, Burger et al. 2015a, Joseph et al. 2015, Jeannerat et al. 2017, 2018). When plastic adjustments to ejaculate quality involves altering the performance of sperm (e.g. sperm velocity or viability) rather than adjustment of sperm number, such adjustments could involve changing the molecular composition of seminal fluid (Perry et al. 2013, Fitzpatrick and Lüpold 2014). Given the influence that SFPs can have on male reproductive success and sperm function they can be expected to be key mediators of adjustment to sperm performance in response to changing levels of sperm competition risk. The role that seminal fluid plays in responses to changing sperm competition risk has yet to be researched extensively in many species, however, the following case studies highlight recent developments and suggest that plastic adjustment of SFPs may have evolved as an adaptation to changing sperm competition risk across a diverse range of taxa.

1.4.2 *Case study 1: Ejaculate plasticity in a bird*

A series of behavioural experiments have been conducted on fowl (*Gallus gallus*) to determine how males of different social status alter ejaculate quality in response to social cues. In this mating system males form a dominance hierarchy in which dominant males gain more copulations than subdominant males because they are preferred by females and will disrupt copulations involving subdominants (Pizzari and Birkhead 2000, Pizzari 2001, Pizzari et al. 2002). Despite attempts to monopolise copulations by dominant males, subdominants are often able to gain some copulation success (Pizzari 2001, Pizzari et al. 2002). Females also vary in quality and honestly advertise quality to males via variation in comb size (Pizzari et al. 2003). Males of different social status have different ejaculate allocation strategies based on levels of sperm competition in addition to the availability and quality of females (Pizzari et al. 2003, Cornwallis and Birkhead 2006). For all males the number of sperm ejaculated decreases with repeated copulations (Cornwallis and Birkhead 2006). When presented with two females, dominant males allocated more sperm to higher quality females, even when mating with that female second, but when presented with females individually sperm number was determined by copulation order (Cornwallis and Birkhead 2006). Subdominant males allocate

high sperm numbers to the initial copulation irrespective of female availability and quality (Cornwallis and Birkhead 2006).

Pizzari et al. (2007) showed that sperm swimming speed, a trait linked to competitive fertilisation success in fowl (Birkhead et al. 1999, Pizzari et al. 2008), was linked to social status and males down-regulated sperm mobility if they remained dominant and up-regulated sperm mobility when they remained subdominant over the course of their experiment. Dominant males were shown to not only allocate more sperm but also faster sperm to high quality females, but their sperm velocity decreased in subsequent copulations, whereas subdominants allocated sperm with similar velocity over subsequent copulations (Cornwallis and Birkhead 2007). Each of these differences in allocation strategy between dominant and subdominant males were shown to be plastic by social manipulation that forced males to switch status (Cornwallis and Birkhead 2006, 2007, Pizzari et al. 2007).

Cornwallis & O'Connor (2009) then demonstrated that adjustments of sperm velocity were influenced by seminal fluid in this system by mixing sperm and seminal fluid from different males. More recent studies have characterised the seminal fluid proteome in domesticated fowl (Labas et al. 2015) and red junglefowl (Borziak et al. 2016), with both studies linking variation in SFPs to either sperm function or male fertility. These studies provide evidence that sperm performance in fowl can be mediated by seminal fluid and in particular SFP composition, however, the exact mechanism that influences sperm velocity remains unknown. Thus, further research is necessary to establish if adjustment of ejaculate quality in response to female quality or social status is linked to SFPs.

1.4.3 *Case study 2: Ejaculate plasticity in a mammal*

Natural populations of house mice (*Mus domesticus*) at high density are characterised by a social structuring in which a single dominant male defends a territory, that usually includes several breeding females and some subdominant males (Bronson 1979). Using genetic markers to determine levels of multiple paternity, Dean et al. (2006) found that litters with multiple sires were more common in higher density populations. Using a similar approach in island populations of house mice, Firman and Simmons (2008) determined levels of multiple paternity and found that relative testis size was greater in populations with higher levels of multiple mating. Further comparison of males from island populations found that males from

more promiscuous populations produced ejaculates containing more sperm, and greater proportions of motile sperm (Firman et al. 2013). These studies demonstrate that male house mice have adapted to sperm competition risk by differentially investing in ejaculates based on the level of multiple mating in different populations.

Furthermore, there is also evidence that male house mice can strategically adjust their investment in ejaculates in response to cues that signal sperm competition risk. Experiments that exposed males directly to rivals and to rival odours, have found that exposure to perceived sperm competition risk results in increased sperm production (Ramm and Stockley 2009, Firman et al. 2013). Exposing males to different densities of rivals as a proxy for sperm competition risk has also been used to demonstrate that males differentially invest in SFPs (Ramm et al. 2015). The expression of three proteins, SVS 5 and SVS 6 that have unknown function but belong to the group of seminal vesicle proteins hypothesised as important in sperm competition (Ramm et al. 2009, 2015), and CEACAM 10 a protein that binds to sperm and enhances sperm motility (Li et al. 2005), was upregulated in the seminal vesicles of males in high sperm competition risk treatments (Ramm et al. 2015).

1.4.4 *Case study 3: Ejaculate plasticity in insects*

For *Drosophila melanogaster*, the ability of males to perceive levels of sperm competition and adjust ejaculate expenditure accordingly is important for their reproductive success, and thus males have evolved to detect the mating status of females (Friberg 2006) and use multiple redundant cues to detect the presence of rivals (Bretman et al. 2011b). Behavioural plasticity in response to the presence of rival males has been well documented, showing that males increase the duration of copulation and achieve a significantly greater share of paternity when in competition with males that were not exposed to rivals, with such responses fully reversible and dependent upon the length of exposure (Bretman et al. 2009, 2010, 2012). Plasticity in SFP production and allocation has also been demonstrated in *D. melanogaster* (refer to section 1.3.2 for discussion of SFPs in *D. melanogaster* and their impact on sperm competition). Males exposed to rivals increased the amount of sex peptide (Acp70A) and ovulin (Acp26Aa) they transferred during mating (Wigby et al. 2009). Exposure to rivals also elicited a reduction in gene expression of Acp62f and Acp26Aa (Fedorka et al. 2011, Mohorianu et al. 2017). Males reared in high density environments allocated relatively greater levels of sex peptide than males reared in low density treatments, possibly in response

to greater perceived sperm competition risk during development (Wigby et al. 2016). In addition to exposure to rivals, males were shown to assess sperm competition risk by determining the mating status of females, altering SFP composition when mating with females that had mated previously compared to virgins (Sirot et al. 2011).

The evolution of ejaculate plasticity has also been highlighted in a series of experiments on Australian field crickets (*Teleogryllus oceanicus*). In this system, sperm viability is the primary determinant of competitive fertilisation success (García-González and Simmons 2005), whereas sperm concentration and morphology have little impact (Simmons et al. 2003). Males produced ejaculates with reduced sperm viability when mating with females that had previously mated with multiple partners (Simmons et al. 2007, Thomas and Simmons 2007) and to virgin females that were coated in cuticular compounds from other males (Thomas and Simmons 2009). Males increased sperm viability following exposure to a single rival male (Simmons et al. 2007), and when reared with exposure to calls from other crickets, respond to acoustic signals in their developmental environment by increasing GSI and sperm viability (Bailey et al. 2010, Gray and Simmons 2013). Simmons and Beveridge (2011) determined that seminal fluid has a significant influence on sperm viability conducting an experiment in which sperm and seminal fluid from different males where separated and recombined. In an experiment using mRNA interference with PCA analysis, Simmons and Lovegrove (2017) then examined levels of gene expression in accessory glands of males reared in different acoustic environments. They found increased sperm viability from males reared with exposure to calls, and increased expression of 7 genes, 3 of which were linked to sperm viability. Reinforcing prior work showing males reduced sperm viability in response to increased sperm competition intensity (Simmons et al. 2007), a recent experiment assessed gene expression in males exposed to high sperm competition intensity (exposure to three rival males in sequence) and found reduced expression across 6 genes including 2 previously linked to sperm viability (Sloan et al. 2018).

Sections 1.2 – 1.4 have provided context for the research conducted in this thesis. The final section of this chapter examines the reproductive biology of my study species the Chinook salmon (*Oncorhynchus tshawytscha*).

1.5 REPRODUCTIVE BIOLOGY OF CHINOOK SALMON

Chinook salmon (*Oncorhynchus tshawytscha*) were introduced to New Zealand from the Sacramento River, California, with successful releases occurring between 1901 and 1907 (McDowall 1994). Several anadromous populations have since established, primarily along the east coast of the South Island in the major braided river systems (McDowall 1994). Although all populations of salmon in New Zealand originate from a single source, variation in phenotypic traits such as egg size, GSI, growth rates and the timing of spawning runs can be observed among populations (Quinn and Unwin 1993, Kinnison et al. 1998, Unwin et al. 2000, Quinn et al. 2001). In addition, there is evidence that some genetic structuring among populations has already developed since introduction (Quinn et al. 2001, Kinnison et al. 2002), although this structure is subtler than that observed for Chinook salmon in their native range between rivers and even seasonal runs within the same river system (Kinnison et al. 2002).

1.5.1 *Salmon life-cycle and alternative reproductive tactics*

Anadromous salmon start their life in freshwater, then migrate to ocean feeding grounds and finally return to freshwater to reproduce (Fleming 1996, Esteve 2005). The time that juvenile fish ("parr") spend in freshwater before migrating to the ocean varies among populations (Unwin et al. 2000) and can range from a few months to a year (Unwin et al. 1999). Additionally, parr can become sexually mature (termed "precocious") without ever leaving the freshwater system (Fleming 1996, Esteve 2005). The time that males spend at sea is also variable, returning between 2 and 7 years of age. In New Zealand, the typical age of return for males is 3-years old, at which males are considered fully grown and are called "hooknoses", reflecting the hooked jaw that develops as a secondary sexual character, while 2-year old returning males are much smaller in size, lack secondary sexual characters and are called "Jacks" (Esteve 2005). Chinook salmon, like most anadromous species, are semelparous- meaning they only participate in a single spawning run before dying, however, a small percentage of precious parr may survive to reproduce again (Unwin et al. 1999).

Variation in the age at which males become sexually mature is the basis for the different life-history strategies and alternative reproductive tactics (ARTs) observed in salmonids. Precocious parr are by far the smallest males in a population of spawning salmon and as such are obligate sneakers (Esteve 2005). Jacks will pair with females if given the opportunity but

are unable to maintain territory in the presence of larger hooknose males (Esteve 2005). As such, jacks almost always adopt a sneaking tactic and, in some cases, develop female colouration as a method to avoid aggression from hooknoses (Esteve 2005). Females also exhibit a preference for hooknose males and will delay spawning when paired with a jack (Berejikian et al. 2000). Hooknose males fight to establish social dominance, with only dominant males able to defend territory around spawning females and thus obtain priority in mating position (Esteve 2005). Subdominant hooknose males that lose contests can either attempt to fight for dominance elsewhere or attempt to sneak fertilisations by invading spawning pairs and releasing their sperm (Esteve 2005). The social status of hooknose salmon is subject to change over the course of a spawning season; for example, in coho salmon (*O. kisutch*), 22% of observed contests between hooknose males resulted in displacement of the previous dominant male (Healey and Prince 1998).

1.5.2 *Sperm competition and reproductive success*

Natural observations have determined that sperm competition occurs in 55-60 % of spawnings (Berejikian et al. 2010, Sørum et al. 2011). In salmonids fertilisation occurs externally as eggs and sperm are released simultaneously into the water (Coward et al. 2002, Esteve 2005). Sperm do not penetrate the egg but rather enter via an opening known as the micropyle, and as such the head of salmon sperm lacks an acrosome (Coward et al. 2002). Fertilisation occurs rapidly, with the majority of eggs fertilised within 10 seconds post ejaculation (Hoysak and Liley 2001, Liley et al. 2002, Yeates et al. 2007). Under these conditions, synchrony of gamete release between male and female is important, and only a two-second delay between the first and second male to release sperm confers a significant advantage to the male that ejaculates first (Yeates et al. 2007). Therefore, dominant hooknose males have a significant reproductive advantage. Firstly, if approximately 40 % of spawning occurs without competition from another male then dominant males monopolise the fertilisation of those eggs. Secondly, through courtship behaviours they can synchronise gamete release with females (Esteve 2005). Thirdly, by occupying priority mating position next to females they can receive a "head start" advantage in sperm competition termed precedence (Berejikian et al. 2010).

Berejikian et al. (2010) conducted an experiment that placed Chinook salmon hooknose and jack males at different densities in artificial waterways made to mimic natural spawning

environments. They observed spawning behaviours and used genetic markers to determine that jacks sired only 20 % of all offspring, despite engaging in a similar number of spawnings. However, for Atlantic salmon estimates using genetic markers reveal that the success of precocious parr can range from 25-89 % at different densities in semi-natural conditions (Morán et al. 1996), and 40-50 % of total progeny sampled from a wild population (Taggart et al. 2001). Mehranvar et al. (2004) placed hooknose sockeye salmon in semi-natural enclosures and found that behavioural indices of dominance are significantly correlated with reproductive success. Nevertheless, dominant traits only explained 33-40 % of the variance in offspring sired, and thus underestimate the reproductive success of subdominant males.

1.5.3 *Ejaculate investment strategies*

As pointed out above, it appears that salmon adopting a sneaking tactic can be successful, even facing a substantial disadvantage relative to dominant hooknose males. A well supported theory is that sneaks compensate for their relatively poor mating position and increased sperm competition risk by increasing their relative investment in ejaculate quality, in particular sperm swimming speed. Several studies confirmed that relative sperm velocity is the primary determinant of competitive fertilisation success in Chinook salmon (Evans et al. 2013, Rosengrave et al. 2016) and other salmonids (Gage et al. 2004, Liljedal et al. 2008, Egeland et al. 2015). A number of studies have found that both jacks and precocious parr make greater investment relative to hooknoses in sperm number (Vladić and Jarvi 2001, Vladić et al. 2002, 2010, Yamamoto et al. 2015), GSI (Butts et al. 2012, Flannery et al. 2013, Makiguchi et al. 2016) and sperm velocity (Flannery et al. 2013, Makiguchi et al. 2016). Additionally, comparison among males with the same life history has shown that secondary sexual characters associated with dominance in salmonids (i.e. red colouration and body size) are negatively correlated with sperm velocity (Pitcher et al. 2009, Yamamoto et al. 2015, 2017, Janhunen et al. 2009). Several studies on Arctic charr (*Salvelinus alpinus*) have shown that males of subdominant social status have faster swimming sperm (Vaz Serrano et al. 2006, Rudolfsen et al. 2006, Haugland et al. 2009, Figenschou et al. 2013). Furthermore, the average delay in sperm release between first and second competitors in Arctic charr was only 0.68 s (Sørum et al. 2011), and further research found this delay resulted in no difference in paternity share between dominant and subdominant males, further supporting that

subdominants may be able to compensate for their poor mating position by producing faster swimming sperm (Egeland et al. 2015).

The ability of hooknose males to adjust ejaculate quality in response to change in social status has only been examined using Arctic charr. In this pioneering study, Rudolfsen et al. (2006) demonstrated that following a social challenge, both sperm concentration and velocity decreased over a four-day period compared with pre-trial levels in dominant males, and also observed an increase in sperm concentration but no change in sperm velocity for subdominant males. However, Rudolfsen et al. (2006) did not evaluate male social status prior to the social challenge, so it is unknown if these males actually changed or simply retained the same status through the course of the experiment. It is also unclear as to how male salmon could adjust sperm velocity over short periods of time and the role that sperm and seminal fluid may play in this process. In guppies (*Poecilia reticulata*), younger sperm achieve superior velocity (Gasparini et al. 2017), thus males could alter ejaculates by influencing the ratio of young and old sperm, by altering the rate of spermiation or turn-over of sperm in the testis. Alternatively, males could adjust seminal fluid components that influence the performance of existing sperm.

1.5.4 *Seminal fluid, SFPs and sperm function in teleost fish*

The role of seminal fluid in maintaining fish sperm in a quiescent state within the testis, and the subsequent changes to seminal fluid osmolality that induce activation of sperm motility upon exposure to water is relatively well understood (Alavi and Cosson 2006, Ciereszko 2008). Studies examining the effect that seminal fluid has on sperm performance during sperm competition in fish, however, are relatively scarce. An exception to this is research using the grass goby (*Zosterisessor ophiocephalus*), that by separating and recombining seminal fluid and sperm from different males, found seminal fluid had a tactic specific effect on sperm velocity, with seminal fluid from sneak males decreasing the velocity of rival guard male sperm and seminal fluid from guard males increasing the velocity of sneak male sperm (Locatello et al. 2013). The underlying molecular mechanism involved has not been identified, although the seminal fluid glycoprotein mucin has been hypothesised as a possible protein involved in this effect (Scaggiante et al. 1999).

The complex role that SFPs play in reproduction, sperm physiology and function for fish has recently become an area of intense research. Experiments that separated SFPs into fractions of different size found that fractions containing proteins < 50 kDa altered sperm velocity and viability in rainbow trout (Lahnsteiner et al. 2004, Lahnsteiner 2007), although the specific proteins involved in this effect have not been identified. The link between SFPs and ejaculate quality has led to several investigations of SFP composition for commercially important teleost species, with traditional (non-proteomic) approaches to protein characterisation using carp (*Cyprinus carpio*) and rainbow trout identifying several major SFPs (Ciereszko et al. 2012). Building on these results, 1D-PAGE prefractionation followed by shotgun proteomic methods were employed for both carp (Dietrich et al. 2014) and rainbow trout (Nynca et al. 2014), identifying 137 and 152 SFPs respectively. Most of the research conducted to date on SFPs in fish has focused on commercial viability and the effect of cryopreservation on semen (Ciereszko et al. 2017).

In the last year, however, two studies have been published investigating seminal fluid in the context of sperm competition in Chinook salmon. In the first, Lewis and Pitcher (2017) describe tactic specific effects of seminal fluid on sperm velocity when separating and recombining seminal fluid and sperm from jack and hooknose males, and found that seminal fluid from jack males significantly decreased the velocity of hooknose male sperm (these results are discussed in Chapter Three). In the second, Gombar et al. (2017) published results of quantified differences in SFP abundance between Chinook salmon jack and hooknose males, identifying 345 SFPs present in both tactics. They found that 21 proteins differed in abundance between the two male phenotypes, including proteins involved in ATP metabolism (L-lactate dehydrogenase B), redox regulation (Superoxide dismutase) and immune function (Precerebellin) that may influence sperm function (these results are discussed in Chapters Four and Five).

1.6 GENERAL AIMS AND SCOPE

The ultimate aim of my thesis research is to use a series of experimental manipulations to determine how Chinook salmon males strategically invest in ejaculate quality when faced with differing sperm competition risk and to explore the role that seminal fluid plays in this process. Historically, studies that have assessed ejaculate quality, particularly for vertebrates, have primarily measured sperm number and sperm traits such as velocity, while seminal fluid

has generally been overlooked. The emerging research that has recently been conducted on seminal fluid provides clear indications that this secretion is a key determinant of ejaculate competitiveness, however, with the notable exception of *D. melanogaster*, the constituents of seminal fluid that alter male reproductive success have not been characterised in detail. For my thesis I investigated strategic adjustment in ejaculate quality, linking sperm performance and seminal fluid protein composition, and the fitness consequences of adjustments made to ejaculates in response to changes in sperm competition risk. I utilise a series of behavioural manipulation, measurements of ejaculate quality, *in-vitro* ejaculate manipulations, *in-vitro* sperm competition fertilisation trials and proteomic analyses.

Each chapter develops a defines set of questions and hypothesis that are assessed independently and will be highlighted in each chapter's preface section. Below is a brief summary of the aims for each chapter and strategies used accomplish them.

Chapter Two aims to use a series of experiments to determine if hooknose male Chinook salmon are able to rapidly respond to changes in social status, that signal changing sperm competition risk, by altering ejaculate quality. The key experiment conducted uses a two-stage social status manipulation that forces males to change their social status, followed by measurement of sperm concentration and computer-assisted sperm analysis (CASA) to measure sperm velocity of ejaculates collected at each experimental stage. Following this, *in-vitro* ejaculate manipulations were employed to determine whether changes to sperm velocity are mediated by seminal fluid, and *in-vitro* fertilisation trials were used to determine if changes to sperm velocity can alter a male's reproductive success under sperm competition conditions.

Chapter Three aims to resolve whether seminal fluid has targeted negative effects on sperm of rival males as previously reported in fish and insects, using Chinook salmon males with different life-histories that adopt ARTs. Utilising a novel approach in Chapter Two, I found that males made investment in seminal fluid of high quality that was beneficial to sperm from any male. In Chapter Three, data from *in-vitro* ejaculate manipulations are used to assess seminal fluid effects on sperm velocity using hooknose and precocious parr males, and data from a recent paper that performed *in-vitro* ejaculate manipulations using hooknose and jack male Chinook salmon are reanalysed using the alternate analysis developed in Chapter Two.

Chapter Four aims to better characterise the Chinook salmon seminal fluid proteome and compare it to published seminal fluid proteomes in teleost fish. A combination of prefractionation techniques, followed by LC-MS/MS analysis and exclusion list searching were used to improve the number of proteins detected when compared with previous studies. Biological function of proteins is assigned using database and literature searching, and the potential functional roles of SFPs in relation to sperm competition are discussed. As a consequence, I provide the first comparative inter- and intra-specific analysis of seminal fluid proteomes in fish.

Chapter Five aims to determine if seminal fluid protein composition is associated with male social status and measures of ejaculate quality. Using seminal fluid samples collected from hooknose males during the social status manipulation reported in Chapter Two, the seminal fluid proteomes of males with different social status and ejaculate quality were analysed using 1D-PAGE prefractionation followed by LC-MS/MS. MS data were then analysed using a spectral counting approach to quantify and compare relative protein abundance.

In summary, this thesis presents a combination of results from multiple experiments showing that Chinook salmon males strategically invest in ejaculate quality in response to sperm competition risk. Collectively, this body of work demonstrates the importance of seminal fluid in the reproductive biology of an externally fertilising fish and contributes to a growing body of literature that highlights the way in which sexual selection drives the adaptive evolution of the entire ejaculate.

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CHAPTER TWO

HOW DO MALES MAKE RAPID ADJUSTMENTS TO SPERM VELOCITY AND DO THESE CHANGES INFLUENCE REPRODUCTIVE SUCCESS?

2.1 PREFACE

*This chapter consists of a published manuscript titled "**Sperm competition risk drives rapid ejaculate adjustments mediated by seminal fluid**" (2017) eLife 6: e28811. The published PDF version of the manuscript can be found in Appendix A.*

As outlined in Chapter One, sperm competition theory predicts that males will make strategic investment in ejaculates with respect to sperm competition risk (Birkhead et al., 2009; Parker, 1998, 1990; Parker and Pizzari, 2010; Wedell et al., 2002). In agreement with this, there is increasing evidence from a range of species that sperm competition risk drives rapid adjustments of ejaculate traits, that occur much faster than the production of new sperm, in response to social cues (Cornwallis and Birkhead, 2007; Kilgallon and Simmons, 2005; Pizzari et al., 2007; Rudolfsen et al., 2006; Smith and Ryan, 2011). This suggests that males may alter the composition of seminal fluid to facilitate such rapid changes in existing sperm (Fitzpatrick and Lüpold, 2014; Simmons and Fitzpatrick, 2012). However, the underlying mechanisms behind changes in ejaculate quality for most species are poorly understood, and research to date has yet to convincingly demonstrate that rapid alterations of sperm quality traits are mediated by seminal fluid.

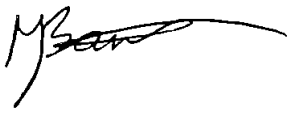
Chapter One also outlines the complex and extremely interesting reproductive biology and life-history variation in salmonids. Fully grown "hooknose" male Chinook salmon (*Oncorhynchus tshawytscha*) engage in intense competition for dominant social status and primary mating position next to spawning females, in a dynamic social environment in which male social status can shift over the course of a spawning season (Healey and Prince, 1998; Esteve, 2005). Male social status is associated with sperm competition risk, as subdominant males must engage in "sneaking" tactics in order to gain access to a female's eggs (Esteve, 2005). As such, the biology of this species makes it ideally suited for the study of male responses in ejaculate quality to changes in sperm competition risk.

This chapter presents the first results of the social manipulation experiment that forms the basis of most of the work presented in my thesis. The overall aim of this experiment was to manipulate male social status, and by recording responses in ejaculate traits, demonstrate the link between social cues that signal sperm competition risk and rapid adjustment of ejaculate investment in Chinook salmon. From there, my aim in this Chapter was to show that

these rapid adjustments are mediated by seminal fluid, and that these changes ultimately influence the outcome of sperm competition and male reproductive success.

2.1.1 STATEMENT OF CONTRIBUTION

As lead author of the following manuscript, I wrote the first and final drafts and designed all figures and tables. Dr Patrice Rosengrave and myself conducted all field work including behavioural manipulations, collection and manipulation of ejaculates, measurement of sperm velocity and sperm concentration, and *in-vitro* sperm competition fertilisation trials. Ilina Cubrinovska conducted the DNA extraction and microsatellite amplification for the paternity analysis. I performed all statistical analyses of the data. I was provided with comments, edits and input for the manuscript from all contributing authors.



Michael J. Bartlett

2.2 SPERM COMPETITION RISK DRIVES RAPID EJACULATE ADJUSTMENTS MEDIATED BY SEMINAL FLUID.

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2.2.1 ABSTRACT

In many species males can make rapid adjustments to ejaculate performance in response to sperm competition risk; however, the mechanisms behind these changes are not understood. Here, we manipulate male social status in an externally fertilizing fish, chinook salmon (*Oncorhynchus tshawytscha*), and find that in less than 48 hours, males can upregulate sperm velocity when faced with an increased risk of sperm competition. Using a series of *in-vitro* sperm manipulation and competition experiments we show rapid changes in sperm velocity are mediated by seminal fluid and the effect of seminal fluid on sperm velocity directly impacts paternity share and therefore reproductive success. These combined findings, completely consistent with sperm competition theory, provide unequivocal evidence that sperm competition risk drives plastic adjustment of ejaculate quality, that seminal fluid harbours the mechanism for the rapid adjustment of sperm velocity and that fitness benefits accrue to males from such adjustment.

2.2.2 INTRODUCTION

Sperm competition (Parker, 1970) occurs commonly across many invertebrate and vertebrate taxa and is a potent evolutionary force influencing male reproductive biology (Birkhead and Møller, 1998; Birkhead and Pizzari, 2002; Simmons and Fitzpatrick, 2012). Sperm competition theory predicts that males will trade-off between energy expended making high quality ejaculates and obtaining mating opportunities, and that males will invest differentially in ejaculates with respect to sperm competition risk (Parker 1990; Parker et al. 1997; Parker 1998; Wedell et al., 2002; Birkhead et al. 2009; Parker and Pizzari, 2010). In agreement with these predictions, males of many species can make rapid adjustments to ejaculate quality within days (Rudolfsen et al., 2006; Pizzari et al., 2007; Thomas and Simmons, 2007; Gasparini et al., 2009; Smith and Ryan, 2011), hours (Cornwallis and Birkhead, 2007a) and even minutes (Kilgallon and Simmons, 2005; Joseph et al., 2015) of exposure to a new social cue that signals changing sperm competition risk, such as the presence of a female, or a male competitor. For

example, in fowl (*Gallus gallus*), males of dominant social status strategically allocate sperm, ejaculating more and faster sperm in initial copulations and to females of higher quality (Pizzari et al., 2003; Cornwallis and Birkhead, 2006; Cornwallis and Birkhead, 2007a; Cornwallis and Birkhead, 2007b), and alter their allocation strategy accordingly when changing social status (Cornwallis and Birkhead, 2007a). While males of several vertebrate species ranging from fish (Rudolfsen et al., 2006; Gasparini et al., 2009; Smith and Ryan, 2011) to humans (Kilgallon and Simmons, 2005; Joseph et al., 2015) can strategically alter the quality of their ejaculate in response to social cues, the mechanism behind such rapid adjustments is as yet unknown.

A promising candidate mechanism for rapid adjustment of sperm velocity may be found in the non-sperm component (seminal fluid and its constituents) of the ejaculate, particularly if such adjustments occur more rapidly than spermatogenesis (Cameron et al., 2007; Perry et al., 2013; Fitzpatrick and Lüpold, 2014). Seminal fluid is a complex medium containing a great diversity of molecules (Poiani, 2006; Juyena and Stelletta, 2012) and is known to influence sperm velocity and motility in vertebrates (Lahnsteiner et al., 1998, 1996; Poiani, 2006; Locatello et al., 2013; González-Cadavid et al., 2014). For example, research using an externally fertilising fish, the grass goby (*Zosterisessor ophiocephalus*), compared males for which sperm competition strategy is determined by age/size and found large males that adopt a guarding strategy have a greater concentration of the seminal fluid glycoprotein mucin (Scaggiante et al. 1999). Furthermore, by separating and recombining seminal fluid and sperm from different males, research using the same species found seminal fluid had a tactic specific effect on sperm velocity, with seminal fluid from sneak males decreasing the velocity of rival guard male sperm and seminal fluid from guard males increasing the velocity of sneak male sperm (Locatello et al. 2013).

However, only one study to date has investigated the role that seminal fluid plays as a mediator of short term plastic sperm performance in a vertebrate species using fowl and the results were inconsistent with theoretical expectation: Cornwallis and O'Connor (2009) found that while ejaculates produced by male fowl that were allocated to females of higher quality contained faster sperm, seminal fluid from those ejaculates reduced the velocity of sperm from the same male allocated to females of lower quality. To be consistent with the prediction that seminal fluid mediates changes in sperm velocity, seminal fluid from ejaculates allocated

to higher quality females should increase, not decrease the speed of sperm isolated from ejaculates allocated to lower quality females. Thus, although there is evidence that seminal fluid can influence sperm velocity, evidence that seminal fluid mediates the rapid plastic adjustment of an ejaculate's motile performance consistent with theoretical expectation is lacking.

We use an ideal model species, chinook salmon (*Oncorhynchus tshawytscha*), to examine patterns of ejaculate plasticity in response to changes in male social status and the reproductive consequences of these changes. In salmonids fertilisation occurs externally and sperm competition occurs in the majority of spawnings (Berejikian et al., 2010; Sørum et al., 2011). Male chinook salmon adopt Alternative Reproductive Tactics (ARTs) situationally, as "hooknose" males fight to establish social dominance (Esteve, 2005). Only dominant males guard spawning females thus obtaining priority in mating position, while subdominant males that lose contests attempt to sneak fertilisations by invading spawning pairs and releasing their sperm (Esteve, 2005). The social status of male salmon is subject to change over the course of a spawning season; for example, in coho salmon (*O. kisutch*), 22% of observed contests between hooknose males resulted in displacement of the previous dominant male (Healey and Prince, 1998). Therefore, in this mating system females mate with multiple males in a dynamic social environment that results in intense levels of fluctuating sperm competition risk.

Previous research has shown that when males engage in sperm competition, sperm swimming speed is the primary predictor of fertilisation success in chinook salmon (Evans et al., 2013; Rosengrave et al., 2016) and other salmonids (Gage et al., 2004; Liljedal et al., 2008; Egeland et al., 2015). Sperm competition theory therefore predicts subdominant males, which have greater sperm competition risk, will invest in ejaculates with faster swimming sperm than dominant males and males changing social status should adjust their investment accordingly (Parker 1990; Parker et al. 1997; Parker 1998; Wedell et al., 2002; Birkhead et al. 2009; Parker and Pizzari, 2010). Indeed, several studies that experimentally manipulated social status using Arctic charr (*Salvelinus alpinus)* have found that subdominant males produce ejaculates with more sperm and faster swimming sperm than dominant males (Liljedal and Folstad, 2003; Rudolfsen et al., 2006; Vaz Serrano et al., 2006 Haugland et al., 2009). Furthermore, Rudolfsen et al., (2006) demonstrated that following a social challenge,

both sperm concentration and velocity decreased over a four-day period compared with pre-trial levels in dominant males, also observing an increase in sperm concentration but no change in sperm velocity for subdominant males. However, Rudolfsen et al., (2006) did not evaluate male social status prior to the social challenge, so it is unknown if these males actually changed or simply retained the same status through the course of the experiment. Recent research shows ejaculates from subdominant Arctic charr sire the same number of eggs when in competition with ejaculates from dominant males if their sperm were released after the average delay observed under natural conditions (Egeland et al., 2015). These results suggest salmonid males in disfavoured mating positions can compensate by producing more competitive ejaculates than dominant males; but whether males changing social status adjust their sperm velocity, and if such adjustments to ejaculates are mediated by sperm or non-sperm components of the ejaculate, is yet to be determined.

Here, we use a comprehensive experimental approach to determine if changes in sperm velocity observed in response to an individual's social position are the result of alterations to the gametes or to seminal fluid and if such responses actually alter a male's reproductive success against a sperm competitor. Specifically, we examine whether ejaculate quality is phenotypically plastic in response to changes in sperm competition risk over 48-hour periods, using a two-stage challenge to manipulate social status (Cornwallis and Birkhead 2007a; Pizzari et al. 2007) and collected ejaculates at each stage of the experiment. In the second stage, males either retained or were forced to change their social status, creating four social phenotypes with varying sperm competition risk (Figure 2.1). We found that subdominant males, which have greater sperm competition risk, invest more in both sperm concentration and sperm velocity compared to socially dominant males. Additionally, we find males that change from dominant to subdominant social status, thus elevated their sperm competition risk, increased their sperm velocity as predicted by sperm competition theory (Parker 1990; Parker et al. 1997; Parker 1998; Wedell et al., 2002; Birkhead et al. 2009; Parker and Pizzari, 2010). We also separated sperm from seminal fluid and created reciprocal combinations both within and between rival males, finding that males can make rapid adjustments to sperm velocity by producing seminal fluid that enhances sperm function. We then used *in-vitro* fertilisation trials and found the seminal fluid effects on sperm swimming speed influences male reproductive success under sperm competition. Our combined experimental results

provide compelling evidence that seminal fluid is the mediator of rapid strategic adjustment of sperm velocity, thus bringing us a critical step closer to identifying the underlying molecular mechanism that enables plasticity of ejaculate performance in dynamic social environments.

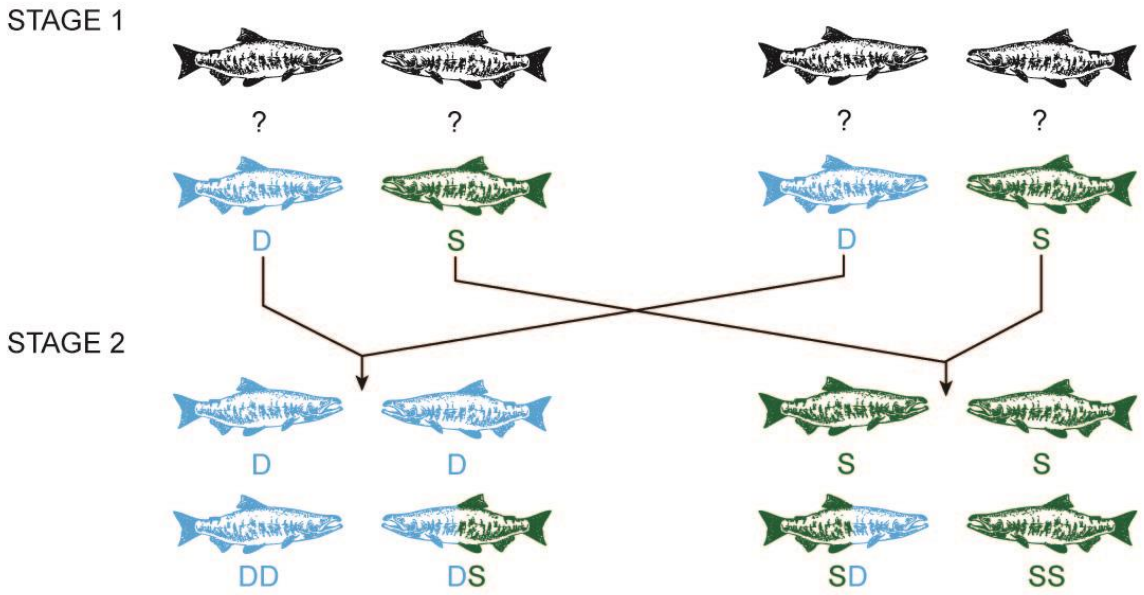

Figure 2.1: Experimental design using a two-stage social status manipulation in chinook salmon. For each trial, in stage 1, four males of unknown social status were used to form two pairs and the social hierarchy within each pairing was then determined, assigning one male as dominant (D) and the other subdominant (S). After 48 hours, ejaculates were collected from each male (D, S, D, S). In stage 2, we reformed pairs, putting males with the same social status together, and re-determined the social hierarchy within each pairing. Males either retained the same status, dominant (DD) or subdominant (SS) in both stages, or changed status in either direction, dominant to subdominant (DS) or subdominant to dominant (SD). After 48 hours, ejaculates were recollected from each male (DD, DS, SD, SS).

2.2.3 MATERIALS AND METHODS

Study species and maintenance

Wild chinook salmon were caught during their annual spawning runs in a trap located on the Kaiapoi River, a tributary of the Waimakariri River system, Canterbury, New Zealand (Unwin et al. 2000). We studied a total of 17 sexually mature 3-year-old females and 44 sexually mature 3-year-old "hooknose" males captured between 27 April and 30 May in 2013, 2014 and 2015. Sample size was informed by related empirical research in this system (Rosengrave

et al., 2008, 2009a). Fish were individually tagged and maintained in a natural river-water raceway (12.5-13°C) at a hatchery (Salmon Smolt NZ, Canterbury, New Zealand) using standard husbandry procedures. All animals were collected and maintained according to the standards of the Animal Ethics Committee for the University of Otago, New Zealand.

Manipulation of male social status

A total of 11 social status manipulation trials were conducted each using four males (n=44; Figure 2.1). On day one, two male dyads were formed pairing males of similar size (average fork length = 71.5 cm, 95% CI = 70.2 - 72.9 cm, n = 44). Each dyad was then placed in a sectioned off part of a river-water raceway (approx. 2.5 m x 2 m x 1 m). Social interactions between the two fish in each dyad were observed for the first day using a series of 10-minute under-water video recordings (GoPro Hero 3), one taken each hour over a 5-hour period, with the first recording starting 15 minutes after introducing fish to the raceway. Male dominance was then determined by calculating a Dominance Index (DI; Winburg et al., 1991; Bailey, 2000; see *Behavioural observations*) using the number of aggressive interactions between males. The male with the higher DI was ranked as dominant (D) and the male with the lower DI as subdominant (S, stage 1 - Figure 2.1). On day two male dyads were left undisturbed and male social status within each dyad established on day one typically remained unchanged (Table 2.1). On day three, male dyads were re-formed placing dominant with dominant and subdominant with subdominant, and a new social hierarchy developed with male social status assigned to each male as described for day one. This forced one fish of each original dyad to change his social status (DS or SD) while the other retained their original status (DD or SS, stage 2 - Figure 2.1). On day four the male dyads were left undisturbed, and the experiment was complete on day five. We determined social status after all the social challenges except in one case where no interaction between males was recorded in the second stage and thus these individuals were excluded from further analyses. A further four males were excluded from analyses due to males escaping from the raceway in the second stage of the experiment, giving a total sample sizes n = 44 in stage one and n = 38 in stage two.

Behavioural observations

Dominance Index (DI) was calculated using the following equation:

DI= Agg+ / (Agg+ + Agg-),

where Agg+ represents the total number of aggressive acts performed and Agg- the total number of aggressive acts received by the individual (Winburg et al., 1991; Bailey, 2000). Aggressive acts were scored using the following criteria:

Charge: Makes a rapid movement towards the other male.

Chase: Continual movement towards the other male with that male actively moving away from aggressor. Each lap around the enclosure from the point where the chase was initiated was scored as one chase, such that continual chasing without pause was scored repeatedly.

Bite: Bites the body of the other male with full gape.

Nip/Nudge: Bites the tail fin of the other male or nudges the other male with a closed mouth.

Table 2.1: The Dominance Index (DI) of the Dominant (D) and Subdominant (S) males in 11 pairings (6 in stage 1 and 5 in stage 2). In 2013 behavioural observations were conducted twice for each pair, on the day the pair was formed (as in other years) and the next day as a means to assess the stability of social hierarchies. We found that in 10 out of 11 pairs the status of males determined on the first day did not change from on the second day.

Social status		**D**	**D**	**S**	**S**
Pair	**Stage**	**Day 1**	**Day 2**	**Day 1**	**Day 2**
1	1	0.844	0.739	0.155	0.26
2	1	0.8	0.75	0.19	0.25
3	2	0.829	0.857	0.17	0.14
4	2	1	0.93	0	0.06
5	1	0.98	1	0.01	0
6	1	0.96	0.89	0.03	0.1
7	2	0.82	0.15	0.2	0.8
8	1	0.97	1	0.03	0
9	1	1	1	0	0
10	2	0.85	1	0.15	0
11	2	1	1	0	0

Measurement of ejaculate quality

Ejaculates were obtained from males by gently applying pressure to the abdomen, taking care to avoid contaminating samples, and was held at 4˚C for up to four hours. We depleted the ejaculate reserves of each male before the experiment, so ejaculates collected later were produced during each 48-hour period. We collected ejaculates in a random order on day three at the end of stage 1 and after social status was manipulated on day five at the end of stage 2 so samples were collected 48 hours after social status was established in each stage.

Sperm velocity measurements were performed in a random order and blind to the social status of each male. We measured sperm swimming speed twice for each male at 10 s post-activation using a CEROS sperm tracker (v 1.2, Hamilton-Thorne Research, Beverly, MA, USA). Approximately 1 µl of milt was activated with river water or ovarian fluid (diluted to 50% with river water) onto a 20 µl Leja slide (Leja Products B.V., Nieuw-Vennep, The Netherlands) on a temperature-controlled stage cooler (TS-4 Thermal Microscope Stage, Physitemp, USA) set to 12.5 °C to match the natural spawning water temperature. We used average path velocity (VAP, µm s^{-1}) as our measure of sperm swimming speed which estimates the average velocity of a sperm cell for 0.5 s over a smoothed path (Rosengrave et al. 2008, 2009a, 2016; Figure 2.2 – Figure Supplement 1). Sperm concentration (sperm/ml) was determined using a Neubauer haemocytometer.

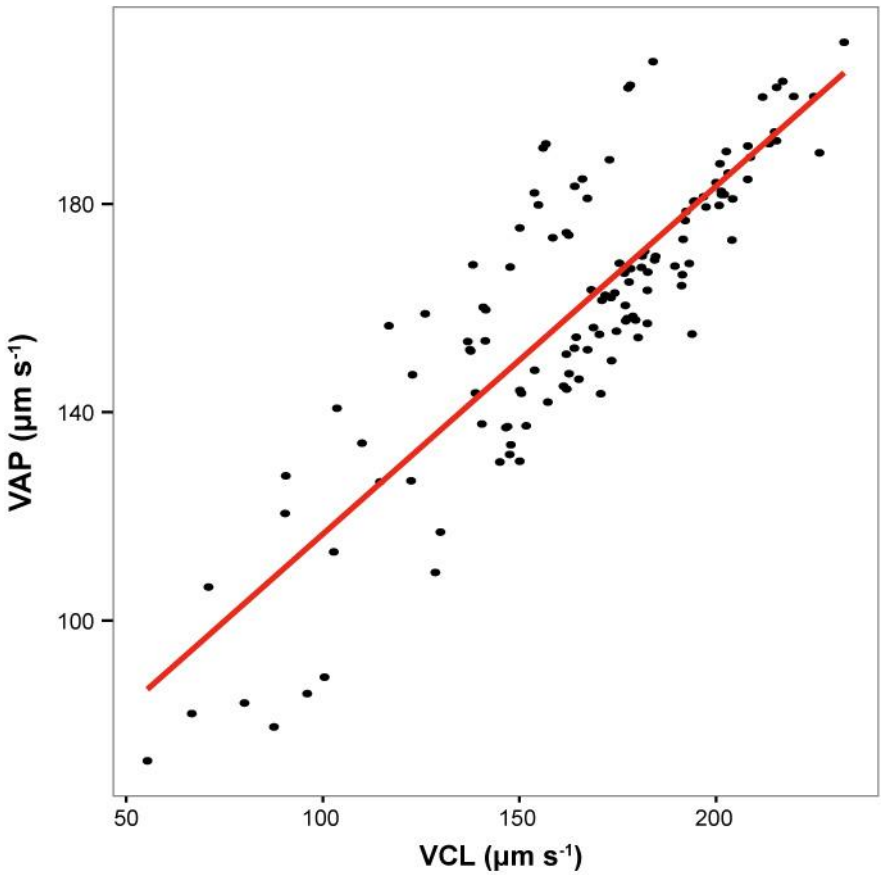

Figure 2.2-Supplement 1: Across all sperm samples collected in this study, Average Path Velocity (VAP) at 10 s post-activation was strongly correlated with Curvilinear Velocity (VCL; r = 0.85, p <0.000l, n = 126). We focused on VAP as an estimate of sperm swimming velocity because we feel that it most closely represents the swimming speed of sperm along a trajectory most likely to encounter fertilizable ova. VAP was calculated from an average 217 (199.1-234.9 95% CI) sperm tracks per milt samples (n = 126 VAP estimates). When calculating difference in VAP we first determined the average VAP for each male using both replicate measures taken at each stage. We used an Intra-class Correlation Coefficient (ICC) and found high agreement between replicates (n = 83, subject variance = 758.7, replicate variance = -0.59, ICC agreement = 0.91) using the package "psy" (RRID:SCR_015660) in R.

Manipulation of ejaculates

To determine the relative roles of sperm and seminal fluid on sperm velocity we centrifugally separate and remix sperm from the seminal fluid of each male with those from the other male in each dyad (n = 42 males in 39 dyads). To prepare recombined ejaculates, milt was centrifuged in 1.5 ml tubes at 4 °C, 300 g for 10 minutes to separate sperm cells from seminal fluid. The seminal fluid was then transferred into a new tube after which 500 µl of artificial seminal fluid (80 mM NaCl, 40 mM KCl, 1 mM $CaCl_2$, 20 mM Tris-HCl) was added to the sperm cells and this was centrifuged again at 4 °C, 300 g for 10 minutes to wash any remaining

seminal fluid from the sperm cells. The artificial seminal fluid was then discarded and recombined ejaculates were prepared using 10 µl of sperm resuspended in 90 µl of seminal fluid from the same male (control) or seminal fluid from their rival, incubated at 12 °C for 20 minutes.

In-vitro fertilisation trials

In 2014 and 2015, at both stages of the social status manipulation trials (Figure 2.1) we conducted a total of 21 replicated *in-vitro* fertilisation trials to determine the effects of ejaculate recombination (seminal fluid) on male fertilisation success. This involved 24 individual males and 17 females in which sperm from the dominant and subdominant male in each dyad competed to fertilise a female's eggs. For each trial, we performed two seminal fluid treatments, using either unmanipulated or recombined ejaculates, in addition to non-competitive controls using sperm from each of the males individually. Haphazardly chosen female fish were killed with a stroke to the head, and their egg batch was expelled through an incision in the abdomen, into a clean bowl. Ovarian fluid was collected by carefully pipetting from each egg batch. Sperm density was adjusted prior to each fertilization trial so that approximately the same number of sperm per male (10^7 spermatozoa) were used in each trial.

For each trial, we placed approximately 100 unfertilized ova from the focal female in a dry 2 l plastic beaker, then added ejaculate samples from each male simultaneously by injecting them separately into a steady stream of raceway water (250 ml at 12.58–138C). This technique simulated natural spawning conditions by facilitating the rapid mixing of eggs with sperm from both males (Rosengrave et al., 2016). We added the ejaculate samples separately into the water to ensure the spermatozoa were activated before the ejaculate samples came into contact, minimizing any effects of each male's seminal fluid on the other male's sperm function. The eggs were allowed to sit for 5 minutes undisturbed until water hardened and were then gently transferred to heath rack trays (12.5-13 °C). We randomly sampled 24 alevins from each replicate fertilisation trial (40 days post fertilisation), placing them in 99% ethanol for DNA extraction and microsatellite genotyping to assess paternity.

DNA extraction, microsatellite amplification and genotyping protocols

To assess paternity share for the males in each sperm competition trial, DNA was extracted from a fin clip for both adult males, the female and 24 offspring from each trial using Chelex®100 resin (Walsh et al. 1991). Three microsatellite loci (Ots 100, Ots 101, Oki 3a; Table 2.2) were then amplified in a multiplex PCR and used to determine paternity by manually matching alleles between offspring, mother and either potential sire. A fourth locus (Ots 104; Table 2.2) was amplified separately using a touchdown PCR protocol and employed when three loci were insufficient to determine paternity without certainty. The genotype of each offspring was always consistent with the expected genotype based on the alleles for the potential sires, i.e. in no offspring did we record unique alleles present for both potential sires.

Table 2.2: Microsatellite primers used to determine paternity. Primers Ots 100, Ots 101 and Oki 3a were amplified in a multiplex reaction, Ots 104 was amplified singly using a touchdown protocol. Letter at 5' end indicates fluorescent label: P = Pet (red), F = Fam (blue), N = Ned (yellow), V = Vic (green).

Primer		Primer Sequence 5'-3'	Master mix	PCR	Source
Ots 100	F	P-tga-aca-tga-gct-gtg-tga-g	Multiplex	Multiplex	Nelson & Beacham (1999)
	R	P-acg-gac-gtg-cca-gtg-ag			
Ots 101	F	F-acg-tct-gac-ttc-aat-tgg-t	Multiplex	Multiplex	Small et al., (1998)
	R	F-tat-taa-tcc-tcc-aac-cca-g			
Oki 3a	F	N-tgt-gct-ata-ggc-tga-atg-tgc	Multiplex	Multiplex	Unpublished, See, Kinnison et al., (2002)
	R	N-aac-aca-ggc-atc-ccc-act-aa			
Ots 104	F	V-gca-ctg-tat-cca-cca-tga	Single	Touchdown	Nelson & Beacham (1999)
	R	V-gta-gga-gtt-tca-ttt-gaa-tc			

Multiplex PCRs were run in 10 µL volume reactions and included the following reagents: 1x PCR buffer (Bioline), 2 mM $MgCl_2$, 0.3 mM dNTPs, 0.4 µM forward and reverse Ots 101 primers, 0.2 µM forward and reverse Ots 100 and Oki 3a primers, 0.5 U of Bioline Taq DNA polymerase, and 0.5 µL of DNA. The thermal cycling conditions for the multiplex protocol

were: 12 minutes at 95°C followed by 10 cycles of 15 seconds at 94°C, 30 seconds at 60°C, and 30 seconds at 72°C, followed by 30 cycles of 15 seconds at 89°C, 30 seconds at 60°C, 30 seconds at 72°C, and a final extension period of 10 minutes at 72°C.

PCRs for amplification of Ots 104 were run in 10 µL volume reactions and included the following reagents: 1x PCR buffer (Bioline), 2 mM $MgCl_2$, 0.3 mM dNTPs, 0.5 µM forward and reverse Ots 104 primers, 0.5 U of Bioline Taq DNA polymerase, and 0.5 µL of DNA. The thermal cycling conditions for the touchdown protocol were: 2 minutes at 95°C followed by 10 cycles of 30 seconds at 95°C, 45 seconds at Ta°C, and 30 seconds at 72°C, where Ta starts at 55°C and drops by 0.5°C each cycle (last cycle should be 50.5°C), followed by 20 cycles of 30 seconds at 95°C, 45 seconds at 50°C, 30 seconds at 72°C, and a final extension period of 10 minutes at 72°C.

PCR samples were genotyped by adding 0.5 µL PCR product to 12 µL HiDi formamide and 0.3 µL Genescan LIZ500 size standard (Applied Biosystems) then run on an ABI3130x1 Genetic Analyser (Applied Biosystems). Results were visualised using GeneMarker v 2.2 (SoftGenetics, RRID:SCR_0156661) and alleles were scored manually.

Statistical analyses

All statistical analyses were performed using R v 3.1.3 (R Core Team, 2016; RRID:SCR_001905). To compare changes in ejaculate quality (sperm velocity (VAP) or sperm concentration) between D and S males, generalised linear mixed effects models (GLMM) were fitted using the package "lme4" (Bates et al., 2015; RRID:SCR_015654). GLMMs using a Gaussian error distribution were fitted using VAP as the response variable, while GLMMs with a Poisson error distribution were fitted using sperm concentration as the response variable. Each GLMM used male social status as a fixed predictor, for stage 1 two levels comparing D and S; and for stage 2, separate models were run with either two levels comparing D and S males with data pooled together (D = D**D** + S**D** and S = S**S** + D**S**), or four levels (males that retained the same status DD and SS, and males that changed status SD and DS). Models with VAP as the response variable used both replicate measurements for each male and included male identity as a random predictor to account for repeated measures.

To test whether males that change social status adjust ejaculate quality, we compared both VAP (GLMMs using a Gaussian error distribution) and sperm concentration (GLMMs with a

Poisson error distribution) in the same males across the two stages of the experiment. Four separate models were run for each of the response variables, separately comparing males in each of the four social phenotypes (DD, DS, SD, SS) and each model used experimental stage (factor with two levels) as a fixed predictor. Additionally, we used an alternative analysis for each of the response variables to test for an interaction effect between social status and experimental stage, both models used social status (factor with four levels; DD, DS, SD and SS), experimental stage (factor with two levels) and the interaction between social status and experimental stage as fixed predictors. Male identity was included as a random predictor to account for repeated measures from the same male.

A linear mixed effects model (GLMM) was fit using the difference in VAP between focal male's sperm recombined with his own seminal fluid and focal male's sperm recombined with his rival male's seminal fluid as the response variable, with difference in VAP between focal male's sperm recombined with his own seminal fluid and rival male's sperm recombined with his own seminal fluid, and social status of rival's seminal fluid as fixed predictors. To fulfil the model's assumption of normality a cube-root transformation was performed on the response variable. We used the random predictors focal male identity, rival male identity and each pairing to account for repeated measures. All VAP measures used were those activated in river water, not ovarian fluid, to avoid female effects on sperm velocity (Rosengrave et al., 2009b, 2016) that could mask the influence of seminal fluid.

To assess the importance of sperm velocity as a predictor of fertilisation success we used a GLMM that was fit using the difference in the number of offspring sired between the focal and rival male in each trial as the response variable, with the relative sperm velocity between males as a fixed predictor. To assess social status as a predictor of fertilisation success we used a binomial GLMM that was fit using the proportion of offspring sired by each male as the response variable, with male social status as a fixed predictor in unmanipulated milt trials and the social status of seminal fluid donor as a fixed predictor in swapped seminal fluid trials. In order to assess the influence of seminal fluid on male fertilisation success we used a GLMM that was fit using the change in the proportion of eggs sired by each focal male across seminal fluid treatments (within the same triad, i.e. within the same male-male-female combination) as the response variable with the change in relative sperm velocity across treatments used as a fixed predictor. For all above models, we used the random predictors focal male identity,

rival male identity, female identity and each unique triad to control for repeated measures. We tested for repeatability of replicate trials conducted for each triad (supplementary material: *Statistical analysis and R code*), removing one triad for which the proportion of eggs sired differed significantly between replicates (n = 20). So that sperm velocity in our model reflected conditions during the fertilisation trials, all VAP measures used were those activated in ovarian fluid, as female effects on sperm velocity can influence the outcome of sperm competition in chinook salmon (Rosengrave et al., 2009b, 2016).

All mentioned models used the week during the spawning season when milt samples were collected as a random predictor to control for potential seasonal effects on milt quality (Butts et al., 2010; Hajirezaee et al., 2010), and the year fish were collected as a covariate (Bolker, 2015). To determine the significance of fixed effects, we present both 95% Confidence Intervals (CI) calculated using the Wald method, and P values calculated for linear mixed effects models with the package "lmerTest" (Kuznetsova et al., 2016; RRID:SCR_015656) using Satterthwaite approximations to calculate degrees of freedom. Assumptions underlying parametric models were verified using residual plots and Shapiro tests. An alpha value of 0.05 was used to evaluate the significance of P-values and adjusted for multiple tests using the Bonferroni method. Refer to Appendix B: *Chapter Two: Statistical analysis and R code*, for all R code used and output from analyses.

2.2.4 RESULTS

Social status and ejaculate quality

Subdominant (S) males had on average faster swimming sperm (Average Path Velocity, or VAP) than dominant (D) males. This difference was not significant when social status was initially determined in stage 1 (Table 2.3; Figure 2.2a) but was significant for stage 2 (Table 2.3; Figure 2.2b). Overall there was considerable variation in sperm swimming speeds among males, accounted for by the random predictor "male identity" that was significant in both stages (stage 1: $\chi^2_{(1)} = 105.11$, $P < 0.001$; stage 2: $\chi^2_{(1)} = 70.02$, $P < 0.001$). Additionally, sperm concentration was significantly higher in S than in D males in stage 1 (Table 2.3; Figure 2.3a), but not stage 2 (Table 2.3; Figure 2.3b). However, sperm concentration for males that remained subdominant (SS) was significantly higher than for those males that remained socially dominant (DD) in stage 2 (Table 2.3).

Table 2.3: Generalised linear mixed effects models (GLMM) to compare sperm velocity (VAP, μs^{-1}) and sperm concentration (cells/ml) among male chinook salmon of different social status (see figure 2.1 for experimental design). In stage 1 of the experiment dominant (D; n = 22) males were compared to subdominants (S; n = 22). In stage 2 separate models were run with the fixed parameter social status with either four levels (males that retained the same status DD (n = 10) and SS (n = 9), and males that changed status SD (n = 9) and DS (n = 10)), or two levels with data pooled together (D = DD + SD (n = 19), S = SS + DS (n = 19)). *P*-values are calculated using Satterthwaite approximations to degrees of freedom and 95% Confidence Intervals were calculated using the Wald method. P-values are adjusted for multiple testing where multiple pairwise comparisons are made using the Bonferroni method with significant values highlighted in bold.

Response variable	**Stage**	**Parameters (fixed effects)**	**estimate**	**95% CI**	***P* value**
VAP	1	Intercept	152.9	135.3 – 170.4	
		D – S	7.4	-8.6 – 23.4	0.37
	2	Intercept	127.1	108.8 – 145.5	
		D – S	19.7	5.1 – 34.2	**0.01**
	2	Intercept	131.2	109.2 – 153.2	
		DD – SS	14.9	-6.5 – 36.5	0.18
		DD – DS	17.9	-2.7 – 38.5	0.09
		SD – DS	24.4	2.9 – 45.9	0.03
		SD – SS	21.5	-0.2 – 43.2	0.06
Sperm concentration	1	Intercept	6.0	5.81 – 6.22	
		D – S	0.2	0.01 – 0.39	**0.04**
	2	Intercept	5.9	5.72 – 6.21	
		D – S	0.2	-0.06 – 0.41	0.14
	2	Intercept	5.8	5.55 – 6.09	
		DD – SS	0.5	0.16 – 0.77	**0.003**
		DD – DS	0.1	-0.16 – 0.43	0.36
		SD – DS	-0.1	-0.44 – 0.18	0.42
		SD – SS	0.2	-0.12 – 0.52	0.21

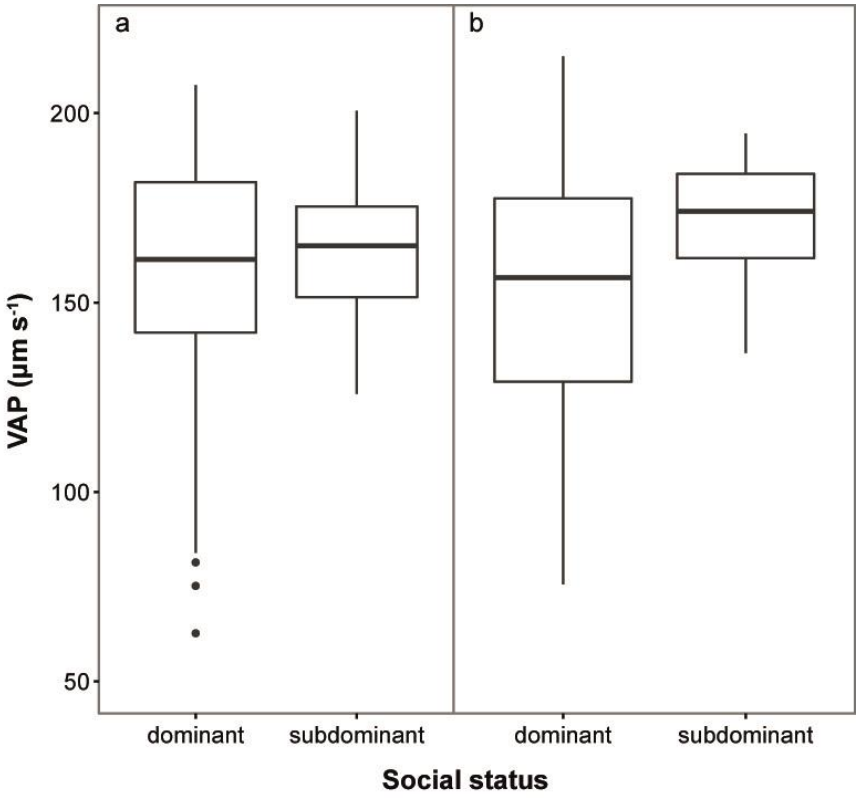

Figure 2.2: Sperm velocity (VAP in µm s^{-1}) in males of dominant (D) and subdominant (S) social status after a: the first social challenge (D, n = 22; S, n = 22) and b: the second social challenge (D, n = 19; S, n = 19). Boxplots display the median of each group with the 25th and 75th percentiles and whiskers extend to data within 1.5 x the inter-quartile range.

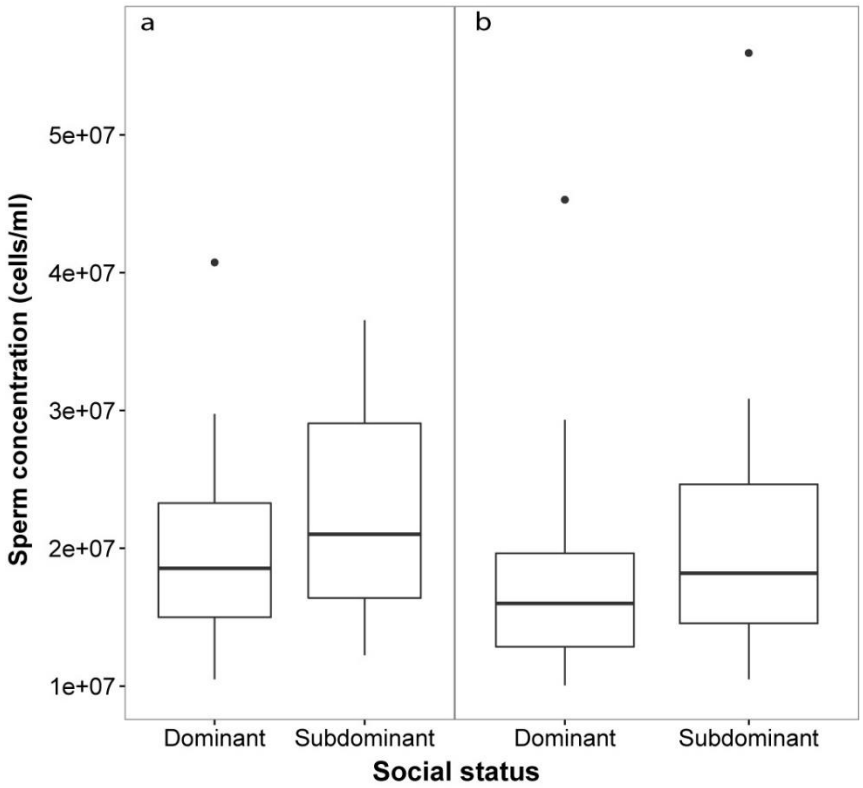

Figure 2.3: Sperm concentration (cells/ml) in males of dominant (D) and subdominant (S) social status after a: the first social challenge (D, n = 22; S, n = 22) and b: the second social challenge (D, n = 19; S, n = 19). Boxplots display the median of each group with the 25th and 75th percentiles and whiskers extend to data within 1.5 x the inter-quartile range.

Ejaculate plasticity in response to social status change

There was a significant increase in mean VAP for males that changed from dominant to subdominant social status (DS; Table 2.4; Figure 2.4). Throughout the social status experiment there were no other significant changes in either VAP or sperm concentration for males of the other social phenotypes (Table 2.4; Figure 2.4). There was also a significant overall interaction effect between social phenotype and experimental stage on VAP ($\chi^2_{(3)} = 11.8$, P = 0.008), with a significant interaction effect found only for males changing from dominant to subdominant status (DS; P = 0.02, 95% CI = 2.9 – 34.9). We found no significant interaction effects between social phenotype and experimental stage on sperm concentration ($\chi^2_{(3)} = 3.0$, P = 0.385).

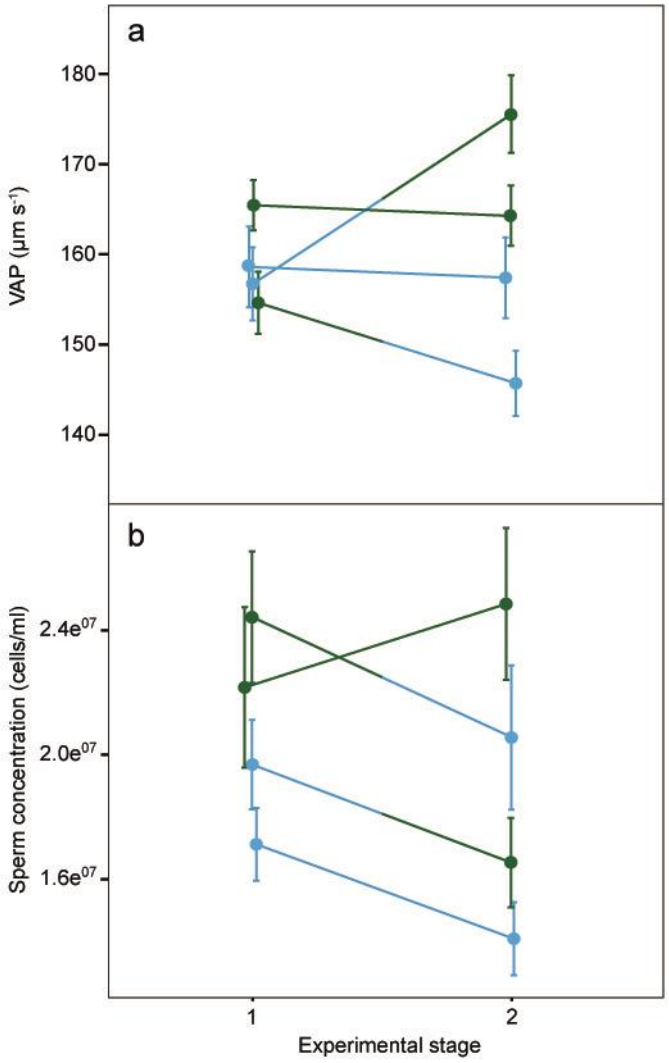

Figure 2.4: Average sperm velocity (VAP, µm s^{-1}; ± s.e.m.) and average sperm concentration (cells/ml; ± s.e.m.) in males of the four social phenotypes after each stage of a social status manipulation experiment in chinook salmon. Blue colour denotes males dominant in both stages (DD, n = 10), green colour denotes males subdominant in both stages (SS, n = 9), a change from blue to green colour denotes males that changed from dominant to subdominant status (DS, n = 10) and a change from green to blue colour denotes males that changed from subdominant to dominant status (SD n = 9). The change in VAP for DS males was statistically significant.

Table 2.4: Generalised linear mixed effects models (GLMM) to compare sperm velocity (VAP, μs^{-1}) and sperm concentration (cells/ml) in males of each social phenotype changing from stage 1 to stage 2 of the experiment. The four social phenotypes are males that remained dominant (DD, n = 10) or subdominant (SS, n = 9) in both stages and males that changed status in either direction, subdominant to dominant (SD, n = 9) and dominant to subdominant (DS, n = 10). *P*-values are calculated using Satterthwaite approximations to degrees of freedom and 95% Confidence Intervals were calculated using the Wald method. P-values are adjusted for multiple testing using the Bonferroni method with significant values highlighted in bold.

Response variable	Social phenotype	Parameters (fixed effects)	estimate	95% CI	P value
VAP	DD	Intercept	109.1	88.9 – 129.2	
		Stage 1 – Stage 2	0.1	-14.1 – 14.4	0.9
	SD	Intercept	139.6	111.9 – 167.2	
		Stage 1 – Stage 2	-8.9	-19.5 – 1.5	0.1
	DS	Intercept	163.9	141.1 – 186.8	
		Stage 1 – Stage 2	17.2	5.4 – 29.1	**0.008**
	SS	Intercept	162.5	147.1 – 177.9	
		Stage 1 – Stage 2	-2.3	-12.0 – 7.4	0.6
Sperm concentration	DD	Intercept	5.6	5.34 – 5.97	
		Stage 1 – Stage 2	-0.2	-0.39 – 0.06	0.2
	SD	Intercept	6.4	6.12 – 6.68	
		Stage 1 – Stage 2	-0.2	-0.48 – 0.002	0.05
	DS	Intercept	6.1	5.56 – 6.58	
		Stage 1 – Stage 2	-0.1	-0.34 – 0.15	0.4
	SS	Intercept	6.4	6.09 – 6.61	
		Stage 1 – Stage 2	0.1	-0.17 – 0.35	0.5

Seminal fluid effect on sperm velocity

Within each dyad, the social status of the rival male was a significant predictor of the difference in VAP between focal male's sperm incubated in their own seminal fluid and the focal male's sperm incubated in their rival's seminal fluid (Table 2.5). Seminal fluid from subdominant males increased the sperm swimming speed of sperm from dominant males, conversely, the seminal fluid from dominant males decreased sperm swimming speed of the sperm from subdominant males (Figure 2.5). However, rival's social status was no longer significant (Table 2.5) when the difference in VAP between the focal male control and rival male control was added as a fixed predictor to the model, for which a significant positive

linear relationship was detected (Table 2.5), with sperm in the seminal fluid of a rival that had faster VAP increasing sperm velocity and sperm in the seminal fluid of a rival that had slower VAP decreasing sperm velocity relative to VAP in their own seminal fluid (Figure 2.6).

Table 2.5: Generalised linear mixed effects models (GLMM) predicting the change in sperm velocity (VAP, μs^{-1}) observed in the focal male's sperm when incubated in either their own seminal fluid or the seminal fluid of their rival male in that dyad, using the social status of the rival male and the relative VAP between sperm from focal and rival males as measured in their own seminal fluid (n = 42 males in 39 dyads). *P*-values are calculated using Satterthwaite approximations to degrees of freedom and 95% Confidence Intervals were calculated using the Wald method. Significant values are highlighted in bold.

Response variable	Model	Parameters (fixed effects)	estimate	95% CI	P value
Change in VAP	1	Intercept	-24.4	-41.8 – -7.0	
		Rival's Social Status	31.4	15.1 – 47.7	**0.0003**
	2	Intercept	-0.64		
		Rival's Social Status	0.44	-0.7 – 1.6	0.465
		Relative VAP	0.05	0.04 – 0.07	**<0.0001**

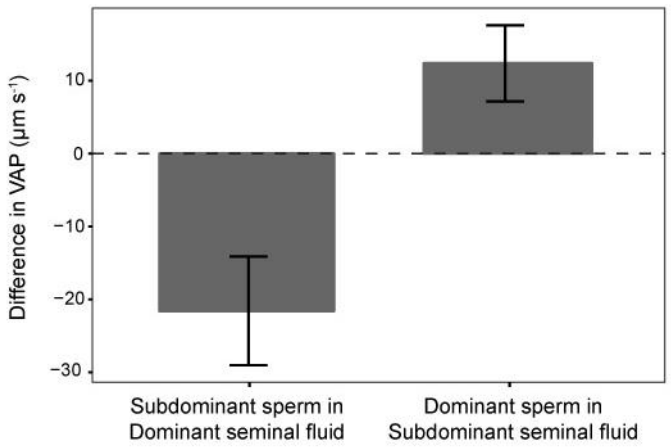

Figure 2.5: Average difference in sperm velocity (VAP, µm s^{-1}; ± s.e.m.) between sperm incubated in their own seminal fluid and incubated in the seminal fluid of their rival in each dyad of a social status manipulation experiment in chinook salmon (n = 42 males in 39 dyads). Seminal fluid from dominant rival males on average decreased VAP of sperm from subdominant males. In contrast, seminal fluid from rival subdominant males on average increased VAP of sperm from dominant males. Social status was a significant predictor of the difference in sperm velocity between sperm incubated in their own seminal fluid and incubated in the seminal fluid of their rival.

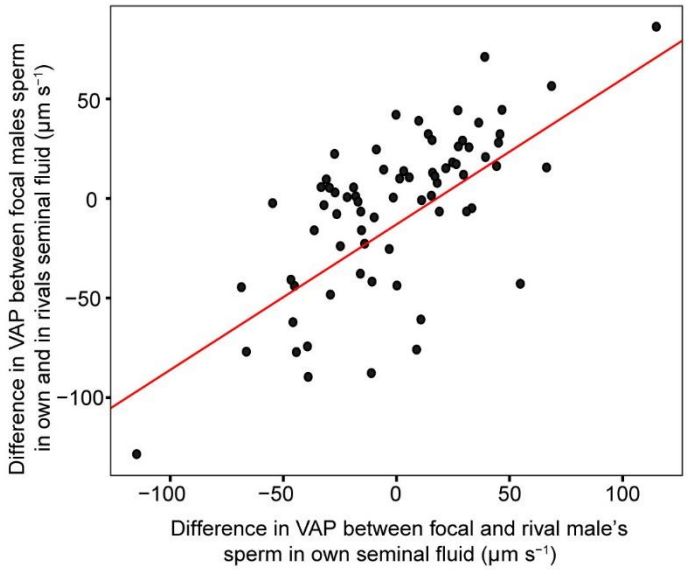

Figure 2.6: Significant linear relationship between the difference in sperm velocity (VAP, µm s^{-1}), between sperm incubated in their own seminal fluid and incubated in the seminal fluid of their rival, and the difference in VAP between sperm from the males in each pairing incubated in their own seminal fluid for each dyad of a social status manipulation experiment in chinook salmon (n = 42 males in 39 dyads). Incubating sperm in the seminal fluid of a rival with faster VAP generally results in an increase in that male's sperm velocity. Likewise, incubating sperm in the seminal fluid of a rival with slower VAP generally results in a decrease in that male's sperm velocity. Raw data is displayed for ease of interpretation, data analysis required transformation (refer to Methods: Statistical analyses and supplementary material for details).

In-vitro fertilisation trials

Male social status was a significant predictor of the proportion of eggs sired (Table 2.6), with subdominant males siring a greater proportion (0.54 ± 0.08 95% CI, n = 21) than dominant males (0.46 ± 0.06 95% CI, n = 21). The social status of the seminal fluid donor when seminal fluid was swapped between males was also a significant predictor of the proportion of eggs sired (Table 2.6), with sperm incubated in the seminal fluid of subdominant males siring a greater proportion (0.6 ± 0.09 95% CI, n = 21) of eggs than sperm incubated in the seminal fluid of dominant males (0.4 ± 0.09 95% CI, n = 21). The difference in sperm velocity between competitors was also a significant predictor of the proportion of eggs sired in both unmanipulated (Table 2.6) and recombined ejaculate seminal fluid treatments (Table 2.6). The change in relative sperm velocity between males within the same male-male-female combinations across seminal fluid treatments was a significant predictor of the change in the proportion of eggs sired by that male's sperm across treatments (Table 2.6; Figure 2.7).

Table 2.6: Generalised linear mixed effects models (GLMM) predicting the fertilisation success of male chinook salmon in sperm competition trials using two males and one female. Trials were conducted using two seminal fluid (SF) treatments, either unmanipulated milt, or recombined ejaculates for which the sperm for both competitors were recombined with the seminal fluid of their rival. Sperm concentration was controlled so that the same number of sperm were used for each male. The first models used the social status of each male to predict the proportion of offspring sired (n = 20). The second models used the relative sperm velocity (VAP, μm s^{-1}) between competitors to predict the difference in offspring sired (n = 20). The final model shows that the change in relative sperm velocity between males within the same male-male-female combinations across SF treatments was a significant predictor of the change in the proportion of eggs sired by that male's sperm across SF treatments (n = 20). *P*-values are calculated using Satterthwaite approximations to degrees of freedom and 95% Confidence Intervals were calculated using the Wald method. Significant values are highlighted in bold.

Response variable	**SF treatment**	**Parameters (fixed effects)**	**estimate**	**95% CI**	**P value**
Proportion of offspring sired	Unmanipulated	Intercept	-0.38		
		Social status	1.11	0.63 – 1.58	**<0.0001**
	Recombined	Intercept	-3.24		
		SF social status	6.23	4.7 – 7.7	**<0.0001**
Difference in number of offspring sired between males	Unmanipulated	Intercept	$-1.49e^{03}$		
		Relative sperm velocity	$1.44e^{-01}$	0.06 – 0.23	**0.003**
	Recombined	Intercept	$3.72e^{03}$		
		Relative sperm velocity	0.13	0.05 – 0.21	**0.003**
Difference in proportion of eggs sired across SF treatments	NA	Intercept	-56.39		
		Difference in relative sperm velocity across SF treatments	0.006	$3.5e^{-03}$ – $7.6e^{-03}$	**0.0001**

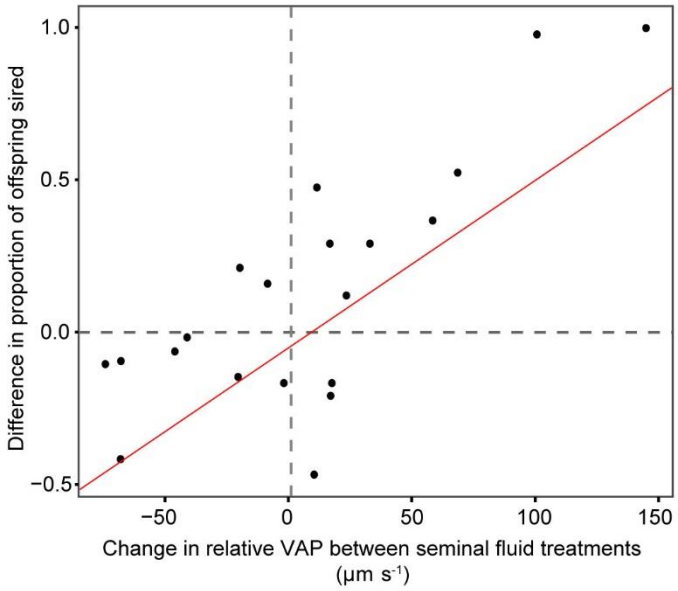

Figure 2.7: Statistically significant relationship between the difference in the proportion of eggs sired by the focal male in each triad from sperm competition trials using chinook salmon (n = 20) when that male's sperm were either incubated in their own or their rival's seminal fluid, and the difference in relative sperm velocity (VAP, μm s^{-1}) between males in each pair when sperm were either incubated in their own or their rival's seminal fluid. The relationship shows that change in fertilisation success across seminal fluid treatments is correlated with the change in relative sperm velocity between competing males in each seminal fluid treatment.

2.2.5 DISCUSSION

In this study, we experimentally manipulated social status to produce four social phenotypes with differing levels of sperm competition risk, and in accordance with sperm competition theory (Parker 1990; Parker et al. 1997; Parker 1998; Wedell et al., 2002; Birkhead et al. 2009; Parker and Pizzari, 2010), found males with the highest risk of sperm competition produced ejaculates with both higher sperm concentration and faster swimming sperm. We also found males can make rapid adjustments to sperm velocity in a strategic response to changes in social position that signal increased sperm competition risk. While seminal fluid is often *implicated* to harbour the unknown mechanism behind plastic sperm performance in vertebrates (Perry et al., 2013; Fitzpatrick and Lüpold, 2014), our combined results for the first time, unequivocally demonstrate that seminal fluid acts as a mediator of rapid strategic adjustment to sperm velocity. Furthermore, we demonstrate strategic adjustments of sperm velocity mediated by seminal fluid directly impact male fitness, highlighting the adaptive significance of plastic ejaculate performance.

Sperm competition theory predicts that males should strategically adjust ejaculates in response to changing sperm competition risk (Wedell et al., 2002; Parker and Pizzari, 2010). In chinook salmon, relative sperm velocity among males is the primary determinant of fertilisation success (Evans et al., 2013; Rosengrave et al., 2016). We show males forced to change from dominant to subdominant social status, and therefore exposed to increased sperm competition risk, responded by increasing the quality of their ejaculate, in this case sperm velocity, within 48 hours (Figure 2.4). While we predict that males forced to change from subdominant to dominant social status, therefore exposed to decreased sperm competition risk, would respond by decreasing their ejaculate quality, we did not see a significant change in sperm velocity for these males. However, subdominant males that later became dominant had a relatively low mean sperm velocity that was more similar to dominant males than those from the other subdominant phenotype in the first stage of the experiment (Figure 2.4). In this case, these subdominant males may have attempted to adopt a guarding tactic even after losing in the first social challenge, as males that lose contests can either sneak or fight for dominance elsewhere (Esteve, 2005).

Males should also strategically adjust sperm concentration in response to changing sperm competition risk (Wedell et al., 2002; Parker and Pizzari, 2010). Accordingly, we found subdominant males produced ejaculates with greater sperm concentration than dominant males. However, our results show that there was no significant increase in sperm concentration for any of the social phenotypes over a 48-hour period. The exact time taken for spermatogenesis in salmonids is unknown, however the process almost certainly takes more than 48 hours (Billard, 1983a; Billard, 1983b; Schulz et al., 2010). Therefore, these results suggest that the observed changes in sperm velocity are mediated by a component of the ejaculate that modifies the competitiveness of existing sperm, rather than simply via the production of new sperm.

Our results clearly demonstrate the observed plasticity of sperm velocity in chinook salmon, a key determinant of fertilisation success in several vertebrate species (Birkhead et al., 1999; Malo et al., 2005; Gasparini et al., 2010; Boschetto et al., 2011) including salmonids (Gage et al., 2004; Liljedal et al., 2008; Evans et al., 2013; Egeland et al., 2015; Rosengrave et al., 2016), is mediated by seminal fluid. We found sperm from the same male, when incubated in seminal fluid from different males, had significantly different sperm velocities, and the direction of

this effect could be predicted by social status. For example, when sperm from dominant males were incubated in seminal fluid from subdominant males we found that on average their sperm velocity increased compared to the baseline measures in their own seminal fluid, and found the opposite effect when sperm from subdominant males were incubated in seminal fluid from dominant males (Figure 2.5). Contrary to Cornwallis and O'Connor (2009), for which seminal fluid from higher quality ejaculates decreased the velocity of sperm from lower quality ejaculates in fowl, our findings are consistent with the prediction that seminal fluid from ejaculates with faster swimming sperm will enhance the speed of sperm from ejaculates with slower sperm. The disparity between our findings and those in fowl (Cornwallis and O'Connor, 2009) possibly reflect differences in the reproductive biology of these species; including internal and external modes of fertilisation and differences in the structure and formation of social hierarchies and associated sperm competition risk.

Ejaculate allocation in fowl is also influenced by factors other than sperm competition risk, including female quality and the probability of future mating opportunities (Pizzari et al., 2003; Cornwallis and Birkhead, 2006; Cornwallis and Birkhead, 2007a; Cornwallis and Birkhead, 2007b); whether such factors influence ejaculate allocation strategies in salmonids is unknown. It is also possible that seminal fluid in fowl has evolved to interact with sperm from rivals, as observed in some insect species (den Boer et al., 2010) and reported for the grass goby (*Zosterisessor ophiocephalus*) (Locatello et al., 2013). Fertilisation occurs rapidly in salmonids, with the majority of eggs fertilised within 10 s post ejaculation (Hoysak and Liley, 2001; Liley et al., 2002; Yeates et al., 2007). Such rapid time frames may allow for little interaction between seminal fluid and sperm from different males during spawning. This is supported by research using Arctic charr that found the activation of sperm with a solution containing seminal fluid from another male had no effect on sperm velocity (Rudolfsen et al., 2015). However, a recent experiment that separated and recombined ejaculates from precocious chinook salmon males (obligate sneakers) and adult hooknose males report similar results to those found in the grass goby, with seminal fluid from precocious males significantly decreasing the velocity of hooknose male sperm (Lewis and Pitcher 2017). Our results suggest chinook salmon seminal fluid has not evolved a targeted effect on sperm from males adopting a different tactic within the same age/size class, as regardless of social status, males that have faster recorded sperm velocities produced seminal fluid that increases the

velocity of sperm from other males with slower speeds, and likewise males with slower sperm velocity produced seminal fluid that decreases the velocity of sperm from males with faster speeds (Figure 2.6).

In addition to demonstrating that seminal fluid influences sperm competitiveness, our *in vitro* sperm competition trials show the influence seminal fluid has on sperm velocity translates to having an effect on male fitness. We measured the fertilisation success within the same male x male x female combinations across trials, and compared those males across unmanipulated and recombined ejaculate treatments, finding changes in the relative sperm velocity between competitors were significantly correlated with the change in the proportion of eggs sired by each male (Figure 2.7). That is, the change in sperm velocity due to the seminal fluid in which sperm were incubated had a significant influence on the proportion of eggs sired by those sperm, in some cases completely reversing the "winner" of sperm competition within the same male-female group. We now need further investigation to determine the component of seminal fluid that is strategically adjusted by males in response to sperm competition risk.

Previous studies have found that natural variation in several seminal fluid metrics was not correlated with sperm velocity in chinook salmon, including pH, osmolality and ion composition (Rosengrave et al., 2009a; Flannery et al., 2013). It is possible that seminal fluid contains different levels of available nutrients therefore fuelling differential energy production in sperm. In the short term following activation of motility in salmonids, sperm utilise ATP as the energy source for flagellar movement (Christen et al., 1987) using both stored ATP reserves and increasing ATP production significantly via aerobic respiration (Lahnsteiner et al., 1993; Lahnsteiner et al.,1999). Sperm ATP levels have been positively correlated with sperm velocity (Lahnsteiner et al. 1998; Bencic et al., 1999; Burness et al. 2004) and fertilisation success (Zilli et al. 2004; Vladić et al. 2010) in external fertilisers. Exposure to different levels of exogenous nutrients in seminal fluid while sperm are immotile in the testis may influence energy metabolism, for example altering available energy reserves or stored nutrient reserves, influencing sperm velocity post activation (Lahnsteiner et al. 1999). Alternatively, seminal fluid may contain peptide or RNA signalling molecules, that alter sperm behaviour. For example, chemotaxis in several marine invertebrates is controlled by signalling pathways that are initiated by chemoattractant peptides released by ova (Kaupp et al., 2003; Darszon et al., 2008; Evans and Sherman 2013). Evidence is also accruing that

proteins and RNAs in seminal fluid exosomes may play critical roles in regulating sperm development and fertilisation (Vojtech et al., 2014; Jodar et al., 2016).

Several Seminal Fluid Proteins (SFPs) have been associated with sperm velocity in vertebrate species (Lahnsteiner et al., 1996, 1998; Poiani, 2006) and are therefore likely candidates for modifying rapid adjustment of sperm velocity (Simmons and Fitzpatrick, 2012). Differences in SFP composition have been documented among males adopting different reproductive tactics in externally fertilising fish (Scaggiante et al. 1999; Gombar et al., 2017). Additionally, a growing body of empirical work has demonstrated that males can tailor SFP composition in response to sperm competition risk (Wigby et al., 2009; Fedorka et al., 2011; Ramm et al., 2015; Simmons and Lovegrove 2017) and the mating status of females (Sirot et al., 2011). The role of SFPs in sperm competition, with the exception for some insect species (den Boer et al., 2010; Avila et al., 2011) and specific proteins in mammals (Ramm et al., 2008), is generally poorly understood. While the activity of SFPs associated with energy metabolism and respiration have been correlated with sperm velocity in a Cyprinid species (Lahnsteiner et al., 1996) and rainbow trout (*O. mykiss*) (Lahnsteiner et al., 1998), total protein concentration as well as the activity of lactate dehydrogenase, anti-trypsin and superoxide dismutase enzymes were not correlated with sperm velocity in chinook salmon (Flannery et al., 2013). However, these SFPs represents only a small fraction of the enzymatic activity likely to occur in fish seminal fluid (Nynca et al., 2014). The critical next step in determining the molecular mechanism(s) involved will be to link variation in seminal fluid components to sperm velocity and confirm these results experimentally.

In conclusion, as predicted by sperm competition theory (Parker 1990; Parker et al. 1997; Parker 1998; Wedell et al., 2002; Birkhead et al., 2009; Parker and Pizzari, 2010), we find male chinook salmon can make rapid adjustment to sperm velocity in response to social cues that signal changing sperm competition risk and such changes have a significant impact on the outcome of sperm competition and therefore male fitness. We further demonstrate that seminal fluid, even in a species with external fertilisation, plays a key role in mediating the strategic rapid adjustment of sperm velocity and for the first time provide strong evidence the mechanism behind plasticity in sperm velocity lies within the non-sperm component of the ejaculate. Our results support plastic adjustment of ejaculate quality in response to

changing sperm competition risk is an effective evolutionary strategy in systems with dynamic social environments and we show seminal fluid mediates such adjustments.

2.2.6 ACKNOWLEDGEMENTS

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2.2.7 ETHICS

Animal experimentation: All animals were collected and maintained according to the approved standards of the Animal Ethics Committee for the University of Otago, New Zealand.

2.2.8 REFERENCES

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CHAPTER THREE

RESOLVING WHETHER TARGETED NEGATIVE EFFECTS OF SEMINAL FLUID ON RIVAL SPERM HAVE EVOLVED IN CHINOOK SALMON

3.1 PREFACE

*This chapter consists of a manuscript in the final stages of preparation for submission to Biology Letters titled "***Can seminal fluid discriminate between sperm from males with alternate reproductive tactics?***".*

A key result from the manipulation of ejaculates performed in Chapter Two (Bartlett et al. 2017) was that the relative difference in sperm velocity between the males in each experimental dyad was a better predictor of the effect that seminal fluid had on sperm velocity than male social status (dominant or subdominant). In other words, males investing in higher quality ejaculates produced seminal fluid that increased the speed of sperm from rival males with lower quality ejaculates, regardless of male social status. This result suggests that seminal fluid does not discriminate between sperm from "hooknose" males with different social status in Chinook salmon (*Oncorhynchus tshawytscha*).

Lewis and Pitcher (2017) also recombined ejaculates, between "jack" and "hooknose" male Chinook salmon and, conversely, found support for a tactic specific effect of seminal fluid on sperm velocity. This result is similar to that reported by Locatello et al. (2013) who recombined ejaculates between sneaker and guard males with different life-histories in the grass goby (*Zosterisessor ophiocephalus*). Both of these studies compared group averages across seminal fluid treatments (i.e., sperm in own seminal fluid *versus* sperm in rival seminal fluid) and found that seminal fluid from sneaker males decreased the velocity of sperm from guards, while having no effect (Locatello et al. 2013) or increasing velocity of sperm from males of the same tactic (Lewis and Pitcher 2017).

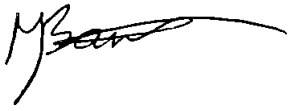
Targeted negative effects of seminal fluid on rival male sperm make evolutionary sense in the grass goby, where sperm and seminal are released in viscous "sperm trails" that dilute slowly into the surrounding water and sperm are motile for an hour or more (Scaggiante et al. 1999, Locatello et al. 2013). In this system, ejaculates have ample opportunity to interact, thus providing opportunity for discriminatory effects or the exploitation of rival ejaculates to evolve (Hodgson and Hosken 2006). In salmonids, however, spawning is characterised by a very short time frame in which the majority of eggs are fertilised within 10 s post ejaculation (Hoysak and Liley 2001, Liley et al. 2002, Yeates et al. 2007). This likely leaves little scope for interaction of ejaculates from different males during spawning.

It is possible that conflicting results between Locatello et al. (2013) and Lewis and Pitcher (2017), and the results in Chapter Two reflect that the former studies compared males with fixed differences in reproductive tactic determined by alternate life-histories, whereas I compared males that adopt reproductive tactics situationally based upon social status. Alternatively, the approaches used to analyse the data (i.e., comparing group averages across seminal fluid treatment groups *versus* testing for a relationship between relative changes in sperm velocity caused by seminal fluid and relative difference in sperm velocity between rival males) may also have resulted in the different conclusions from these studies with those of Chapter Two.

In this chapter, I aimed to resolve whether targeted negative effects have evolved in Chinook salmon by manipulating ejaculates from males with alternate life-histories, early maturing "precocious parr" males and fully grown "hooknose" males using a fully crossed design and compared the analytical approaches described above. Additionally, I recently reached out to Trevor Pitcher and Jason Lewis, and requested that we collaborate on this manuscript. They agreed, and have generously provided me with their data, which has been reanalysed and included in this chapter. This manuscript was ready for submission, but is now awaiting comments and feedback from Trevor Pitcher and Jason Lewis following the additional reanalysis of data from Lewis and Pitcher (2017).

3.1.1 STATEMENT OF CONTRIBUTION

As lead author of the following manuscript in preparation, I wrote the first and final drafts and designed all figures. Dr Patrice Rosengrave and myself conducted all field work including collection of ejaculates, manipulation of ejaculates and measurement of sperm velocity. Associate Professor Trevor Pitcher and Jason Lewis generously provided their data for analysis. I performed all statistical analyses of the data. I was provided with comments, edits and input for the manuscript from Dr Patrice Rosengrave and Associate Professor Tammy Steeves.



Michael J. Bartlett

3.2 CAN SEMINAL FLUID DISCRIMINATE BETWEEN SPERM FROM MALES WITH ALTERNATE REPRODUCTIVE TACTICS?

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3.2.1 ABSTRACT

Mounting evidence demonstrates the importance of seminal fluid influencing sperm function in response to sperm competition risk. Some studies suggest that males invest in seminal fluid that decreases the performance of rival sperm, whereas others indicate investment in "high quality" seminal fluid that improves the performance of all sperm. Resolving whether seminal fluid can indeed discriminate between own and rival sperm is critical for elucidating mechanisms underlying seminal fluid effects on sperm competitiveness. Here, using Chinook salmon (*Oncorhynchus tshawytscha*), we experimentally manipulated ejaculates recombining sperm and seminal fluid from males with different alterative reproductive tactics (ARTs), and find that the relative difference in sperm velocity between paired males, regardless of ART, predicted changes in sperm velocity caused by seminal fluid. Our findings suggest that selection favours investment in components of the seminal fluid that influence all sperm similarly, rather than having detrimental targeted effects on sperm from males with rival mating tactics. Thus, we argue that studies need focus on *relative* changes in sperm performance, to provide key insight on the evolution of sperm traits mediated by seminal fluid.

3.2.2 INTRODUCTION

Sperm competition is a widespread phenomenon in most taxa and occurs when the sperm of rival males compete to fertilise the same group of eggs (Parker 1970). A common adaptation to sperm competition risk is differential investment in ejaculate quality; producing greater numbers of sperm (delBarco-Trillo 2011, Kelly and Jennions 2011) and/or altering sperm traits associated with reproductive success, for example sperm velocity or viability (Snook 2005,

Fitzpatrick and Lüpold 2014). Evidence is now accruing that differences in ejaculate competitiveness among males may also be attributed to seminal fluid rather than intrinsic differences in sperm (Fitzpatrick and Lüpold 2014, Poiani 2006, Simmons and Fitzpatrick 2012, Perry et al. 2013), but for many systems the proximate mechanisms by which seminal fluid acts to alter sperm performance are poorly understood.

Knowledge of mechanisms is vital in producing accurate predictions using sperm competition theory, and thus improving our understanding of how ejaculate allocation strategies have evolved (Parker and Pizzari 2010). However, before mechanisms involved can be elucidated, it is critical to establish whether seminal fluid has targeted effects that can discriminate between sperm. On one hand, males may invest in "high quality" seminal fluid that has similar effects on sperm from any male, such as improving the survival of both own and rival sperm (Holman 2009). Alternatively, seminal fluid could have targeted negative effects on rival ejaculates that decreases the performance of competitor's sperm. For example, there is convincing evidence in social insects demonstrating seminal fluid can incapacitate sperm from rival males (den Boer et al. 2010, 2015).

Few examples of such targeted negative effects have been successfully reported outside of social insects; however, two experiments using externally fertilising fish with alternate reproductive tactics (ARTs) suggest that seminal fluid has targeted negative effects, decreasing the velocity of sperm from males of the alternate tactic (Locatello et al. 2013, Lewis and Pitcher 2017). In one of these experiments, our recent manipulation of ejaculates from Chinook salmon (*Oncorhynchus tshawytscha*) males with different life-histories, we compared average sperm velocity across treatment groups, and found that seminal fluid from males with the higher sperm competition risk tactic decreased velocity of sperm from males of the lower risk tactic (Lewis and Pitcher 2017). While these results align with sperm competition theory, we expect that targeted seminal fluid effects would be limited in salmonids due to rapid spawning that leaves minimal time for seminal fluid and sperm from different males to interact.

Recently we found that when experimentally manipulating male social status/sperm competition risk in "hooknose" Chinook salmon, seminal fluid mediated rapid adjustments to sperm velocity and consequently competitive fertilisation success (Chapter Two; Bartlett et al. 2017). However, while we could predict the effect of seminal fluid on sperm velocity using

social status, a better predictor was the relative sperm velocity between males, suggesting a quality driven rather than targeted effect (Chapter Two; Bartlett et al. 2017). These results are contrary to those comparing males with different life-histories (Lewis and Pitcher 2017) and suggest that accounting for the relative sperm velocity of males in each pairing can reveal patterns that are not apparent when comparing averages across treatment groups.

Here we use Chinook salmon males with different life histories adopting fixed ARTs that experience different levels of sperm competition; small early maturing "precocious" males that must sneak fertilisations, and larger "hooknose" males that gain primary access to spawning females (Esteve 2005). In a fully crossed design, we experimentally manipulate ejaculates from males with different ARTs. We examine whether seminal fluid has targeted negative effects on the velocity of sperm from rival males by comparing averages across treatment groups (Locatello et al. 2013, Lewis and Pitcher 2017). Using an alternate approach, we then test for investment in high quality seminal fluid by comparing relative sperm velocity of rival males with changes in sperm velocity caused by rival seminal fluid, using both data collected in this experiment and reanalysing data from our previous experiment in Chinook salmon that compared group averages only (Lewis and Pitcher 2017).

3.2.3 MATERIALS AND METHODS

Wild three-year-old hooknose male Chinook salmon (n=5) were caught in 2016 during their annual spawning run in a trap located on the Kaiapoi River, Canterbury, New Zealand. One-year-old precocious males (n=6) were obtained from a hatchery population (Salmon Smolt NZ, Canterbury, New Zealand) located on the same river tributary. Fish were individually tagged and maintained in a natural river-water raceway (12.5-13°C) at the hatchery using standard husbandry procedures. Milt was obtained from males, in a random order, as previously described (Rosengrave et al. 2016, Chapter Two; Bartlett et al. 2017). All samples were held at 4˚C for a maximum of four hours before sperm motility analyses were conducted.

To test the effects of seminal fluid on sperm performance from hooknose and precocious males, each male's sperm was centrifugally separated and recombined with seminal fluid (Chapter Two; Bartlett et al. 2017) from; the same male (control), and from each male of the other life history in a random order (recombined; total of 57 recombined ejaculates). Three

of the 60 possible recombinations were not performed because there was not enough ejaculate available from the precocious male.

We measured sperm velocity for each male at 10 s post-activation using a CEROS sperm tracker (v 1.2, Hamilton-Thorne Research, Beverly, MA, USA). For each male two measurements were taken twice for each ejaculate treatment; unmanipulated ejaculate (milt), control (males own seminal fluid separated from sperm and then recombined) and recombined (combined with seminal fluid of opposite mating tactic). Approximately 1 µl of ejaculate was activated with river water onto a 20 µl Leja slide (Leja Products B.V., Nieuw-Vennep, The Netherlands) on a temperature-controlled stage cooler (TS-4 Thermal Microscope Stage, Physitemp, USA) set to 12.5 °C. We used average path velocity (VAP, µm s^{-1}) as a measure of sperm velocity as this sperm trait correlates with reproductive success in this species (Rosengrave et al. 2016, Chapter Two; Bartlett et al. 2017).

All statistical analyses were performed using R v 3.1.3 (R Core Team 2017). Generalised Linear mixed effects models were fit with "lme4" (Bates et al. 2015) using male identity and each pairing as random predictors to control for repeated measures. P values were calculated with the package "lmerTest" using Satterthwaite approximations to calculate degrees of freedom. Refer to Appendix C: *Chapter Three: Statistical analysis and R code*, for all R code used and output from analyses.

3.2.4 RESULTS

Centrifugation resulted in a significant decrease in VAP, which was consistent across both reproductive tactics (centrifugation, t = -2.5, d.f. = 31, p = 0.017, 95% CI = 2.72 – 21.47; tactic x centrifugation, t = 0.6, d.f. = 31, p = 0.549, 95% CI = -16.69 – 8.77; Figure 3.1a). Comparison of control with recombined treatments showed no overall significant effect on sperm velocity when sperm were incubated in seminal fluid of a male with different tactic, however there was a significant tactic x treatment interaction (treatment, t = 1.06, d.f. = 36.9, p = 0.293, 95% CI = -6.54 – 22.13; tactic x treatment, t = -4.11, d.f. = 40.4, p < 0.001, 95% CI = -56.94 – -20.17; Figure 3.1b). Post-hoc analysis revealed that seminal fluid from hooknose males resulted in a significant reduction in velocity for precocious male sperm (t = 4.55, d.f. = 37, p < 0.001; Figure 3.1b), while precocious seminal fluid had no significant effect on hooknose male sperm (t = -1.06, d.f. = 37, p = 0.293; Figure 3.1b).

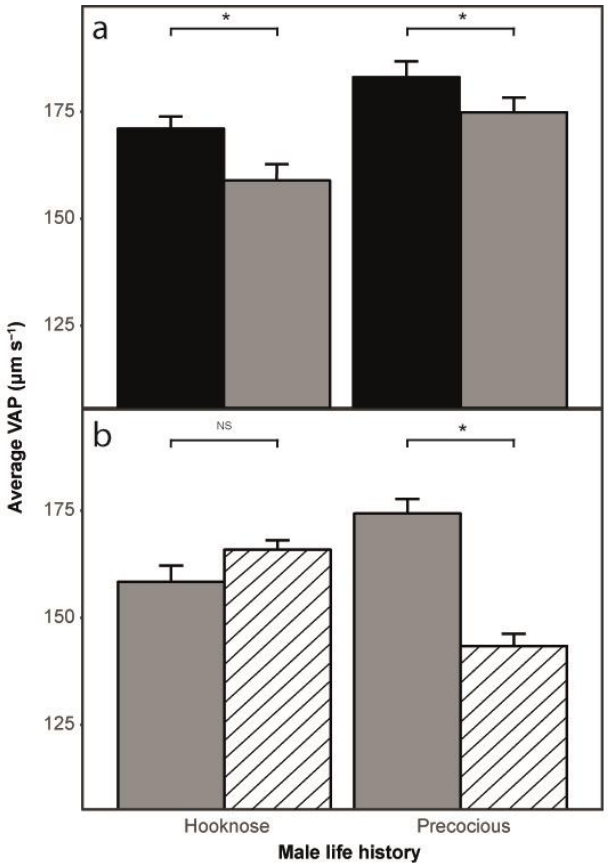

Figure 3.1: Average (± standard error) sperm velocity (VAP, µm s^{-1}) in males with different life-histories in Chinook salmon comparing a) unmanipulated milt (black bars) with sperm resuspended in own seminal fluid (Control, grey bars), and b) sperm resuspended in own seminal fluid (Control, grey bars) with sperm resuspended in the seminal fluid of male from other life history (Recombined, striped bars). An asterisk (*) denotes a significant post-hoc test ($p < 0.05$).

We found that the difference in sperm velocity between the males in each pair (control treatment) was a significant predictor of the change in sperm velocity when sperm were incubated in the seminal fluid of the other male in each pair ($t = 3.45$, d.f. = 36.2, $p = 0.001$, 95% CI = 0.24 – 0.86; Figure 3.2a). Reanalysis of data from Lewis and Pitcher (2017) also found a significant correlation between these variables ($t = 2.95$, d.f. = 17.1, $p = 0.009$, 95% CI = 0.11 – 0.57; Figure 3.2b).

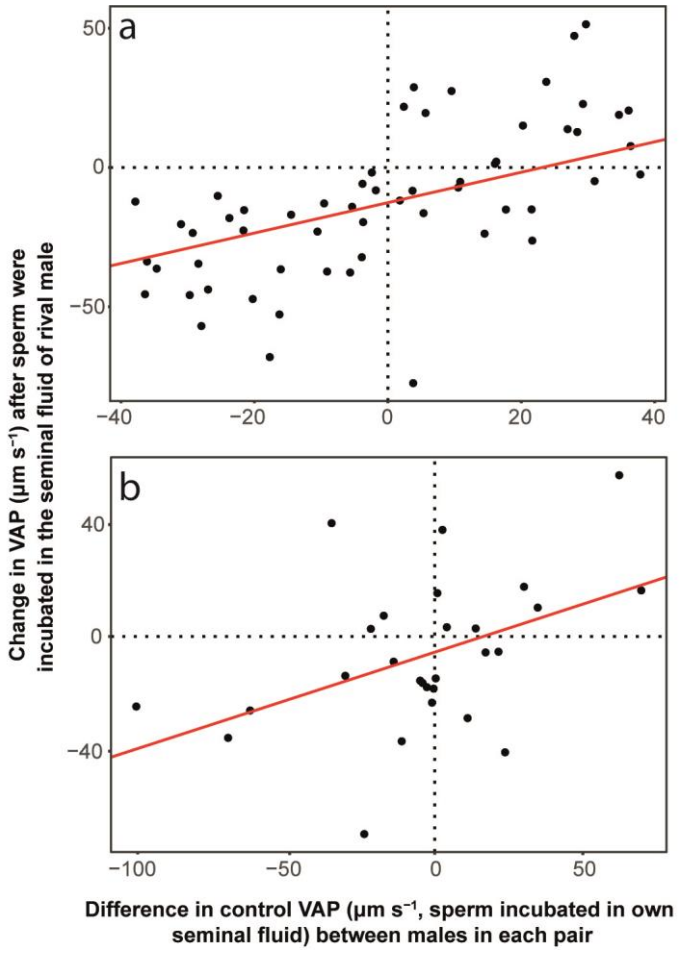

Figure 3.2: Significant linear relationship between the change in sperm velocity (VAP, µm s^{-1}) after sperm were incubated in the seminal fluid of their rival male with different mating tactic relative to sperm incubated in their own seminal fluid (control), and the difference in VAP for control treatment between sperm from the males in each pairing in a) pairs of Chinook salmon males from this experiment (n = 5 hooknose and 6 precocious males, 57 recombined ejaculates) and b) pairs of Chinook salmon males from a previous experiment (n = 14 males of each tactic, 28 recombined ejaculates) (Lewis and Pitcher 2017).

3.2.5 DISCUSSION

Increasing evidence, including results presented here, shows that seminal fluid impacts key sperm traits influencing male reproductive success. Comparing average sperm velocity across treatment groups supported a tactic specific effect of seminal fluid on sperm velocity; however, we also found that the change in sperm velocity when incubated in another male's seminal fluid was significantly correlated with the relative sperm velocity between males in each pair. These results support a quality driven rather than tactic specific effect.

Further supporting this, reanalysis of data from a previous experiment that manipulated ejaculates in Chinook salmon males with ARTs and reported tactic specific effects (Lewis and Pitcher 2017) also found a significant correlation between change in sperm velocity caused by rival seminal fluid and relative sperm velocity between rival males. While our results do not rule out the possibility that tactic specific effects may have evolved in external fertilisers with ARTs, combined with those from our recent experiment on hooknose Chinook salmon (Chapter Two; Bartlett et al. 2017), they do suggest that comparing averages between treatment groups (Locatello et al. 2013, Lewis and Pitcher 2017) may mask patterns that are revealed only by comparing individuals in each pairing when sperm and seminal fluid are recombined.

Collectively, the results from this study and those from Chapter Two (Bartlett et al. 2017) assesses data from males of all three life-history strategies in Chinook salmon and strongly suggests that targeted negative effects on rival sperm have not evolved in this species. This likely reflects the exceptional set of conditions that characterise sperm competition in salmonids, where sperm must find and fertilise an egg within an incredibly short time frame in an external environment (Hoysak and Liley 2001, Yeates et al. 2007). These conditions provide strong selection for fast swimming sperm while also likely constraining the evolution of interaction between ejaculates from different males, preventing both discriminatory effects against rivals and the exploitation of rival ejaculates.

While the interactions between sperm and seminal fluid are likely to differ among species, in particular those with internal and external fertilisation, our results further highlight the need for careful analysis of seminal fluid effects on sperm function. Indeed, truly resolving targeted versus quality driven effects in many cases may require the application of innovative

methods; for example re-examining incapacitation of rival sperm via seminal fluid in *Drosophila melanogaster* by staining sperm (Manier et al. 2010). Detailed understanding of mechanisms underlying ejaculate allocation strategies will improve model predictions and advance sperm competition theory (Parker and Pizzari 2010). Moving forward, resolving quality versus targeted effects of seminal fluid on sperm function in many taxa will be key to the discovery of proximate mechanisms behind sperm and seminal fluid interactions.

3.2.6 ACKNOWLEDGEMENTS

We are grateful to the hatchery staff at Salmon Smolt, New Zealand, in particular Karl French, Errol White and Luke Price. We are also grateful to Fish and Game, North Canterbury, New Zealand, in particular Dirk Barr, for invaluable logistical support**.** We thank Ilina Cubrinovska for field assistance

3.2.7 ETHICS

Animal experimentation: All animals were collected and maintained according to the approved standards of the Animal Ethics Committee for the University of Otago, New Zealand.

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CHAPTER FOUR

DESCRIPTIVE PROTEOMICS OF CHINOOK SALMON SEMINAL FLUID AND COMPARISON AMONG TELEOST SPECIES

4.1 PREFACE

This chapter consists of analyses and results for a manuscript in preparation for submission to a Special Issue in Molecular & Cellular Proteomics. The final manuscript will combine results from this chapter with the quantitative proteomic analyses from Chapter Five.

Proteomic methods that aim to characterise entire protein complements have emerged as powerful tools in the study of reproduction, ecology and evolution (Karr 2008, Findlay and Swanson 2010, Diz et al. 2012, Baer and Millar 2016). The application of proteomics is particularly powerful for studies of reproductive systems, where proteins mediate a number of key processes including sperm-egg interactions, sperm competition, postmating prezygotic isolation and conflict between sexes (Findlay and Swanson 2010, Sirot et al. 2015, McDonough et al. 2016). Proteomic approaches to study the complex role that proteins play in reproduction, sperm physiology and function for fish has recently become an area of intense research (for review see Ciereszko et al. 2017). To date, three studies have been published describing seminal fluid proteomes for teleost fish species, these are discussed in further detail throughout this chapter.

By manipulating ejaculates in Chapters Two and Three, separating and recombining sperm and seminal fluid from different individuals, I have shown that males with greater sperm competition risk invest in high quality seminal fluid that has a significant influence on sperm velocity. Given the influence that proteins in the seminal fluid can have on sperm performance (reviewed in Chapter One), proteins are the ideal candidates for eliciting seminal fluid effects on sperm velocity in this system. The overall aim of the proteomic work conducted as part of this thesis research, was to identify candidate proteins involved in sperm and seminal fluid interaction that may mediate seminal fluid effects on sperm velocity in Chinook salmon (*Oncorhynchus tshawytscha*). However, proteomic methods to quantify differences in protein expression invariably lead to the production of a list of identified proteins (Laukens et al. 2015). A considerable amount of work must be made to interpret the information in such lists that involves distilling large amounts of information from databases and available literature (Laukens et al. 2015).

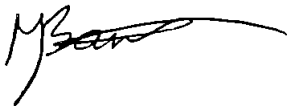
In this chapter, I take the opportunity to present a descriptive analysis of the proteome list generated by this thesis research. I discuss functional groups of proteins identified in the

context of sperm competition and sperm physiology in external fertilisers. Using methods to compare protein sequences with the available protein lists for teleost species, I present the first comparative inter- and intra-specific analysis of seminal fluid proteomes in fish.

Given the size of the full list of proteins identified would be impractical to include as a table in the printed thesis, an electronic supplementary file has been provided (Supplement 1: full protein list) that will eventually be available has online supplemental material in the published manuscript.

4.1.1 STATEMENT OF CONTRIBUTION

As lead author of the following manuscript in preparation, I conducted all laboratory work while under supervision of Dr Julia Grassl and Prof. Boris Baer. Dr Patrice Rosengrave and myself conducted all field work. Dr Julia Grassl performed the Mascot searches. I performed all subsequent analyses of the data, including processing of the Mascot output, literature and database searching, comparisons among proteome lists and GO analysis. I wrote the first and final drafts and designed all figures and tables. I was provided with comments, edits and input from all contributing authors.



Michael J. Bartlett

4.2 CHARACTERISING THE SEMINAL FLUID PROTEOME IN CHINOOK SALMON (*ONCORHYNCHUS TSHAWYTSCHA*)

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4.2.1 ABSTRACT

The non-sperm component of ejaculates, seminal fluid, has important biological functions during reproduction. Recent evidence shows that in an externally fertilising fish, the Chinook salmon (*Oncorhynchus tshawytscha*), males invest in seminal fluid that increases sperm velocity and consequently male reproductive success when under high threat of sperm competition. Seminal fluid proteins (SFPs) are thought to be likely mediators of sperm performance, however the seminal fluid proteome of teleost fish has not been characterised to the extent that it has in some other vertebrate species. To improve upon our knowledge of the seminal fluid proteome in Chinook salmon, we employed a combination of prefractionation techniques, followed by LC-MS/MS analysis utilising an exclusion list method to produce a high confidence list of 549 SFPs, identifying 378 proteins not reported previously in this species. We find a significant overlap between our results and the proteome lists produced in previous studies on reproductive fluids in teleost fish, suggesting conserved function across species. Detailed GO analysis revealed that most proteins have metabolic functions, with 25 % involved in the metabolism of proteins and amino acids. Proteins that function in energy metabolism, defence against reactive oxygen species and in signalling pathways linked to the regulation of sperm motility were detected and are discussed in the context of sperm competition.

4.2.2 INTRODUCTION

An ejaculate is made up of sperm and a non-sperm component, seminal fluid, the latter of which is now recognised as playing a pivotal role in reproduction (Poiani 2006, Perry et al. 2013, McGraw et al. 2014). In external fertilisers, such as many teleost fish, ejaculates and eggs are simultaneously released into the water where seminal fluid appears to provide a favourable microenvironment for sperm function (Billard 1986, Ciereszko 2008). Prior to

ejaculation, seminal fluid is excreted into the spermatic duct providing an optimal environment for the storage sperm, and in the case of salmonids, keeping sperm immotile for months prior to spawning (Ciereszko 2008, Ciereszko et al. 2013). Research also indicates that seminal fluid also plays an important role during sperm competition (reviewed in Perry et al. 2013), when ejaculates of multiple males compete to fertilise a female's eggs (Parker 1970).

For example, males of the externally fertilising Chinook salmon (*Oncorhynchus tshawytscha*) have been found to produce seminal fluid that increases sperm velocity, a key trait influencing reproductive success, when experiencing higher sperm competition risk (Lewis and Pitcher 2017; Chapter Two (Bartlett et al. 2017) & Chapter Three (Bartlett et al. in preparation)). Furthermore, in Chinook salmon and other externally fertilising fish species, female ovarian fluid released with the eggs can also influence sperm velocity, and has been implicated as a mediator of cryptic female choice (Dietrich et al. 2008, Rosengrave et al. 2008, 2009, 2016, Egeland et al. 2016, Alonzo et al. 2016). However, the exact molecular mechanisms and interactions between sperm and both seminal and ovarian fluid components that alter sperm velocity, and ultimately sperm competitiveness, remain largely unknown.

Seminal fluid is a medium containing a variety of different molecules (Poiani 2006), and within this complex mixture, seminal fluid proteins (SFPs) have been identified as probable mediators of sperm performance under sperm competition conditions (Simmons and Fitzpatrick 2012). The activity of enzymes involved in energy metabolism, including pyruvate kinase, lactate dehydrogenase and malate dehydrogenase has already been correlated with sperm velocity in a Cyprinid species (*Alburnus alburnus*) and the rainbow trout (*Oncorhynchus mykiss*) (Lahnsteiner et al. 1996, 1998). Additionally, fractions containing SFPs < 50 kDa in size alter sperm velocity and viability in rainbow trout (Lahnsteiner et al. 2004, Lahnsteiner 2007), although the specific proteins involved in this effect have not been identified. The link between SFPs and key sperm traits has stimulated a number of investigations to determine the proteomic composition of seminal fluid for two commercially important teleost species, common carp (*Cyprinus carpio*) and rainbow trout. Initially, traditional approaches to protein characterisation that purify and then sequence individual proteins identified several major SFPs (Ciereszko et al. 2012).

Building on these results, 1D-PAGE prefractionation followed by shotgun proteomic methods were employed for both common carp (Dietrich et al. 2014a) and rainbow trout (Nynca et al.

2014), identifying 137 and 152 SFPs respectively, with "substantial" overlap between the proteins identified (Ciereszko et al. 2017). Most recently, Gombar et al. (2017) quantified differences in SFP abundance between Chinook salmon (*Oncorhynchus tshawytscha*) males with different life-histories that adopt alternative reproductive tactics, large "hooknose" males that guard territory around females, and smaller "jack" males that attempt to sneak fertilisations. Jack males typically make greater investment in ejaculates, producing more sperm and faster sperm, than hooknose males (Flannery et al. 2013, Lewis and Pitcher 2017). Gombar et al. (2017) identified 345 SFPs present in both tactics and found that 21 proteins differed in abundance between the two male phenotypes, including proteins involved in energy metabolism (L-lactate dehydrogenase B), redox regulation (Superoxide dismutase) and immune function (Precerebellin) that may influence sperm function.

While research to date represents a significant improvement in our knowledge of SFP composition in externally fertilising fish, the number of proteins identified remains relatively low compared with other species, for example in human seminal fluid 923 (Pilch and Mann 2006) and 1,487 (Milardi et al. 2012) SFPs have been identified and 1,141 SFPs were identified in red jungle fowl (Borziak et al. 2016). While a lack of total genome sequencing and annotation for many teleost species limits identification of SFPs (Ciereszko et al. 2017), an additional challenge when working with body fluids, is the large dynamic range of protein abundances (Wu and Han 2006). Here, to detect as many proteins as possible in Chinook salmon seminal fluid, we employed a combination of 1D-PAGE and offline HPLC prefractionation techniques followed by LC-MS/MS analysis utilising an exclusion list method (Eubel et al. 2008). This method enabled us to characterise the seminal fluid proteome in detail and compare with previous proteomes from reproductive fluids of teleost fish, discussing the function of SFPs and their potential role in sperm competition.

4.2.3 MATERIALS AND METHODS

Study species and Seminal fluid collection

Wild chinook salmon were caught during their annual spawning runs in a trap located on the Kaiapoi River, a tributary of the Waimakariri River system, Canterbury, New Zealand (Unwin et al. 2000). We studied a total of 17 sexually mature 3-year-old "hooknose" males captured between 27 April and 30 May in 2014. Males were subject to a two-stage social status

behavioural manipulation experiment in which they were paired and social status (dominant or subdominant) was assessed at each stage, after which seminal fluid samples were collected using abdominal massage (Chapter Two; Bartlett et al. 2017).

Seminal fluid samples for proteomic analysis were prepared from milt collected at both experimental stages (n = 17 males, 34 samples, see Chapter Two, Figure 2.1 for social manipulation experimental design) and stored on ice within 10 minutes of collection. Milt was centrifuged at 4 °C, 300 g for 20 minutes to separate sperm from seminal fluid. This initial slow speed was used to minimise damage to sperm and therefore release of cellular proteins into the seminal fluid. The supernatant was then centrifuged at 4 °C, 20 000 g for 20 minutes, frozen in liquid nitrogen and stored at -80 °C prior to further analysis.

Sample prefractionation and digestion

Protein concentration of each sample was determined using a Bradford assay. Each sample (30 µg) was loaded onto 1D SDS-PAGE gels. Two gels were run in total, each gel included a sample for each of the 17 males. The first gel ran samples collected during stage one of the social status manipulation and the second gel ran samples collected during the second stage of the social status manipulation. Each lane of the gels was cut out using a scalpel and the < 50 kDa section (Figure 4.1) was used for LC-MS/MS analysis. The samples were destained and dehydrated with acetonitrile, gel pieces were then allowed to swell in 50 µL of 1 ng/ µl trypsin (Promega) in 10mM ammonium bicarbonate, on ice for 30 minutes, before incubating at 37 °C overnight. Samples were resuspended in 50 µL of 5% HPLC acetonitrile/0.1% formic acid (v/v) before passing through 0.22-mm centrifugal filters (Millipore) to remove any gel pieces (Figure 4.2a).

Additionally, a second prefractionation method was employed to maximise the number of proteins detected. Seminal fluid was pooled from 8 samples, selecting one male at random from each of the four possible social phenotypes for samples collected in both the first and second stage of the social status manipulation. In total, 200 µg of protein was treated with 10 mM DL-dithiothreitol for 30 min followed by 100 mM iodoacetamide for 30 min in the dark. This was digested overnight at 37 °C in 50 µL of 1 ng/µl trypsin (Promega). Salt removal and pre-fractionation by high pH, reversed-phase fractionation (Yang et al. 2012) was performed off-line on an Agilent 1200 series HPLC configured with two J4SDS-2 guard columns (PolyLC)

and an XBridge™ C18 3.5 µm, 4.6 × 250 mm column (Waters). Peptides were loaded onto the C18 column in 5% (v/v) acetonitrile/10 mm ammonium formate (pH 10/NH_4OH) before separation by 5–60% (v/v) acetonitrile gradient at 1 ml/min in 1 min windows over 96 min in a 96-deep well plate in a row by row fashion. Individual wells that showed high peptide concentrations (n = 16) in the chromatogram were analysed separately. The remaining wells were pooled orthogonally as columns of the 96-well plate resulting in 12 fractions as detailed in Yang et al. (2012). All fractions and individual wells were dried down in a vacuum centrifuge, resuspended in 50 µL of 5% HPLC acetonitrile/0.1% formic acid (v/v) before filtered through 0.22-µm centrifugal filter units before MS analysis (Figure 4.2b).

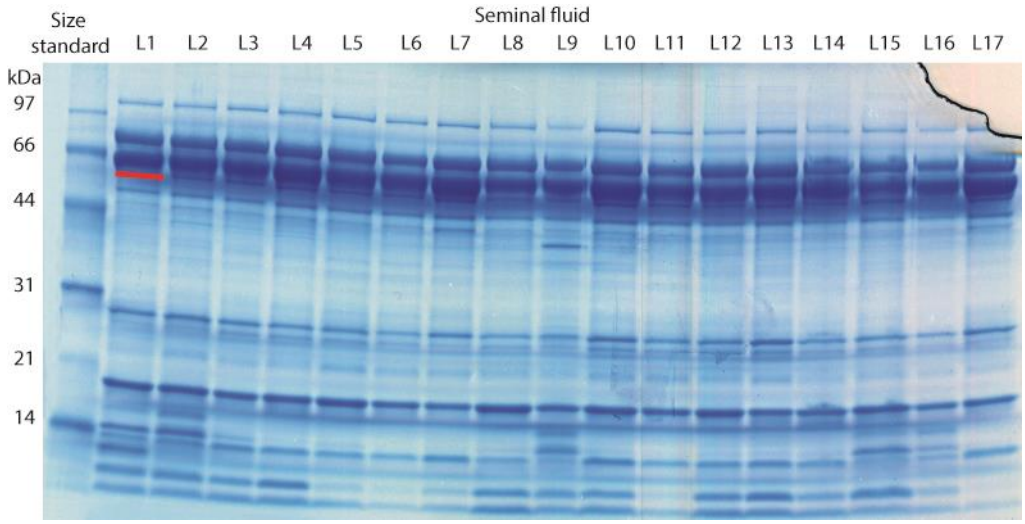

Figure 4.1: One dimensional separation of chinook salmon seminal fluid proteins by SDS-PAGE. Everything below the red line was included in LC-MS/MS analysis.

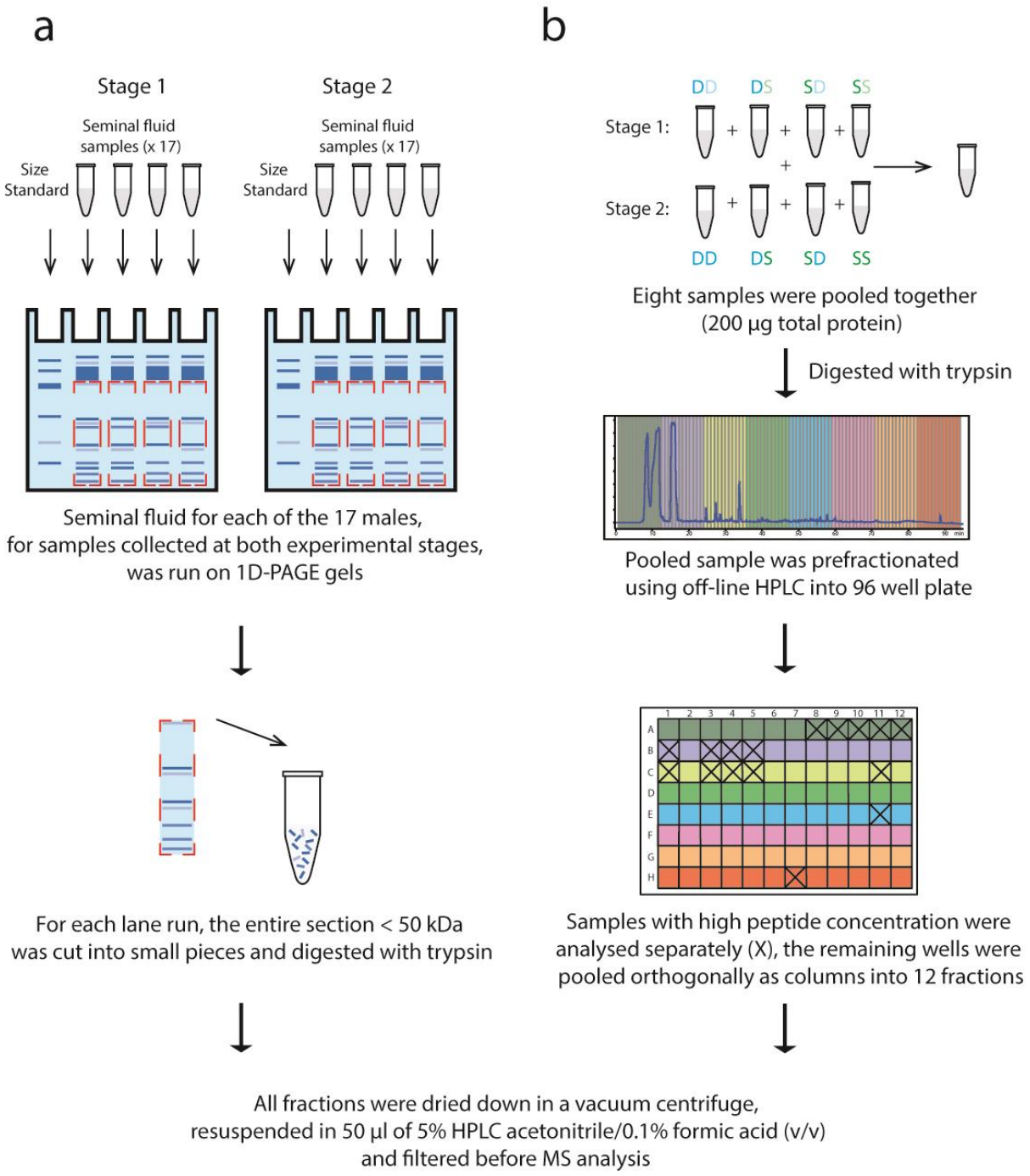

Figure 4.2: Prefractionation methods employed in this study: a) seminal fluid samples from both experimental stages for 17 males were prefractionated using 1D-PAGE and the < 50 kDa section of each lane was used for MS analysis. b) Seminal fluid from eight males was pooled together, one from each of the social phenotypes across both experimental stages, and prefractionated using off-line HPLC.

LC-MS/MS analysis

LC-MS/MS was performed with samples analysed in quadruplicate (8 µg per analysis) on an Agilent 6550 Q-TOF with Chip Cube interface and C18 trapping/analytical Polaris chip using 45 min of 10% to 30% (v/v) acetonitrile gradients in 0.1% (v/v) formic acid. Settings used were positive ion mode, eight mass spectrometry (MS) scans at 250 to 1,400 mass-to-charge ratio per second, maximum of eight precursors per cycle with an absolute threshold of 5,000, scan speed varied according to abundance, and charge state selection set to +2 and +3 and selected by abundance.

Database searching, results filtering and functional annotation

Spectra from all MS runs were pooled and searched against a combined Salmoninae and common contaminant database (17,639 and 58 sequences respectively) using Mascot 2.5.1 (Matrix Science). The following parameters were used: (i) enzyme: trypsin; (ii) fixed modification: Carbamidomethyl (C); (iii) variable modifications: Oxidation (M); (iv) peptide tolerance: 100 ppm; (v) MS/MS tolerance: 0.5 Da; (vi) peptide charge: +1, +2, and +3; (vii) instrument: ESI-QUAD-TOF; and (viii) allowing up to one missed cleavages. To increase the number of identifications, the peptide list generated from Mascot was exported and then used as an exclusion list (based on the peptide (m/z) and charge (z)) for a repeat the Q-TOF run of the same fraction (Eubel et al. 2008). We used the decoy function within Mascot to calculate a false discovery rate (FDR) of 2% and a minimal a score of 27. We then filtered this list by taking only a single representative protein accession for proteins grouped by Mascot into families. For proteins where only a single peptide was identified, MS/MS spectra were examined by eye and proteins with low peptide ion scores and poor b-, y-ion series were removed from further analyses.

In order to compare the overlap between our protein list with those from previous studies, we used two methods to directly search our protein sequences against those from other studies. The programme FASTA (Pearson and Lipman 1988) was used to compare protein sequences alignments and Mascot was used to search spectra generated in this study against databases compiled from the protein lists (using a conservative minimum Mascot score of 40) for seminal fluid proteomes of rainbow trout (Nynca et al. 2014), carp (Dietrich et al. 2014a) and Chinook salmon (Gombar et al. 2017) as well as the ovarian fluid proteomes of Chinook

salmon (Johnson et al. 2014) and rainbow trout (Nynca et al. 2015). Additionally, to identify proteins in our list potentially excreted into the seminal fluid as part of extracellular vesicles, we compared our list to a list of 94 of the top 100 exosome associated genes from ExoCarta (Keerthikumar et al. 2016) with protein sequences obtained from UniProtKB (www.uniprot.org; The UniProt Consortium 2017) for Euteleostomorpha species.

The UniProtKB database was used for Gene Ontology (GO) annotations for "biological process" and "molecular function". For unassigned proteins, annotation was made for "biological process" using BLAST searches against the UniProtKB database, assigning GO for each protein on annotation of the best BLASTp matching sequence where available. Next, we searched Web of Science (www.webofknowledge.com) using the protein name/description and the keywords "seminal fluid", "semen", "sperm" and "reproduction" assigning GO for each protein based on description of biological function in published research. To predict the subcellular location of the identified proteins, we used TargetP 1.1. The location assignment predicts the presence and location of signal peptide cleavage sites in amino acid sequences (Emanuelsson et al. 2000). Proteins previously described in teleost seminal or ovarian fluid were classified as extracellular, and we further classified proteins as secreted if their localization was described as extracellular within KEGG (Kanehisa et al. 2007), or UniProtKB.

4.2.4 RESULTS

A total of 1097 proteins with a minimum Mascot score of 27 (FDR 2%) were detected. After filtering for protein families, 740 proteins remained and a further 179 proteins detected with only a single peptide were removed. Post filtering, we present a high confidence list of 549 proteins identified in Chinook salmon seminal fluid (Supplement 1: full protein list). We improve upon the proteome for seminal fluid in Chinook salmon, identifying 378 proteins not reported previously (Figure 4.3a). Nearly half (49%) of the 346 SFPs detected previously in chinook salmon (Gombar et al. 2017) were matched to proteins from this study (Figure 4.3a). There was also substantial overlap with proteomes reported for rainbow trout (90%) (Nynca et al. 2014) and common carp (64%) (Dietrich et al. 2014a) (Figure 4.3b). Combining results from Gombar et al. (2017) with our own brings the current total number of non-redundant SFPs detected in Chinook salmon seminal fluid to 723 proteins.

Of the 549 proteins we identified, 298 (54%) were classified as extracellular proteins. A total of 524 (95%) were assigned GO classification for biological process and 385 (70%) were assigned molecular functions. Proteins were then sorted into 13 broad biological process categories and 8 molecular function categories (Figure 4.4a). Nearly half (47%) of the identified proteins are associated with metabolic processes, with 25% involved in the metabolism of proteins and amino acids. Of the remaining proteins, 13% were involved with defence, against microorganisms (10% immune function) and reactive oxygen species (3% ROS defence). Our study of the molecular function of these proteins revealed that 59% of proteins identified had either binding or catalytic activities (Figure 4.4b). Consistent GO profiling with previous research combined with the highest Mascot scoring proteins representing abundant SFPs previously detected in teleost's (Nynca et al. 2014, Dietrich et al. 2014a, Gombar et al. 2017), further supports that seminal fluid proteomes are highly conserved among teleost species and the proposed major functional roles of seminal fluid in teleost species; to protect spermatozoa within the testes and during fertilisation, and to regulate key physiological processes such as energy metabolism and motility (Ciereszko 2008, Ciereszko et al. 2013).

The available information in the literature and UniProtKB for proteins assigned to the "Metabolism (Protein and AA)", "Metabolism (DNA and RNA)" and "Regulatory" broad biological process categories was used to further sort proteins into more informative sub-categories (Figure 4.4c). Nearly half (67 [49%]) of the 136 proteins involved in the metabolism of proteins and amino acids were part of protein degradation or proteolysis pathways (includes proteolysis inhibitors) (Supplement 1: full protein list). We also found 12 were involved in protein phosphorylation, 7 proteins that regulate cell motility, 25 proteins involved in energy metabolism, and 18 proteins involved in protecting sperm from oxidative stress (Table 4.1). Additionally, we found that 75% (70 of 94) of the most commonly expressed proteins detected in exosomes are represented in chinook salmon seminal fluid.

Many of the proteins identified in both chinook salmon (Johnson et al. 2014) and rainbow trout (Nynca et al. 2015) ovarian fluid were also detected in chinook salmon seminal fluid (85% - 89% respectively; Fig. 4.3c). There were 27 proteins detected only in ovarian fluid that do not appear to be related to sperm velocity, including proteins involved in egg development, for example vitelline envelope proteins and chorion constituents.

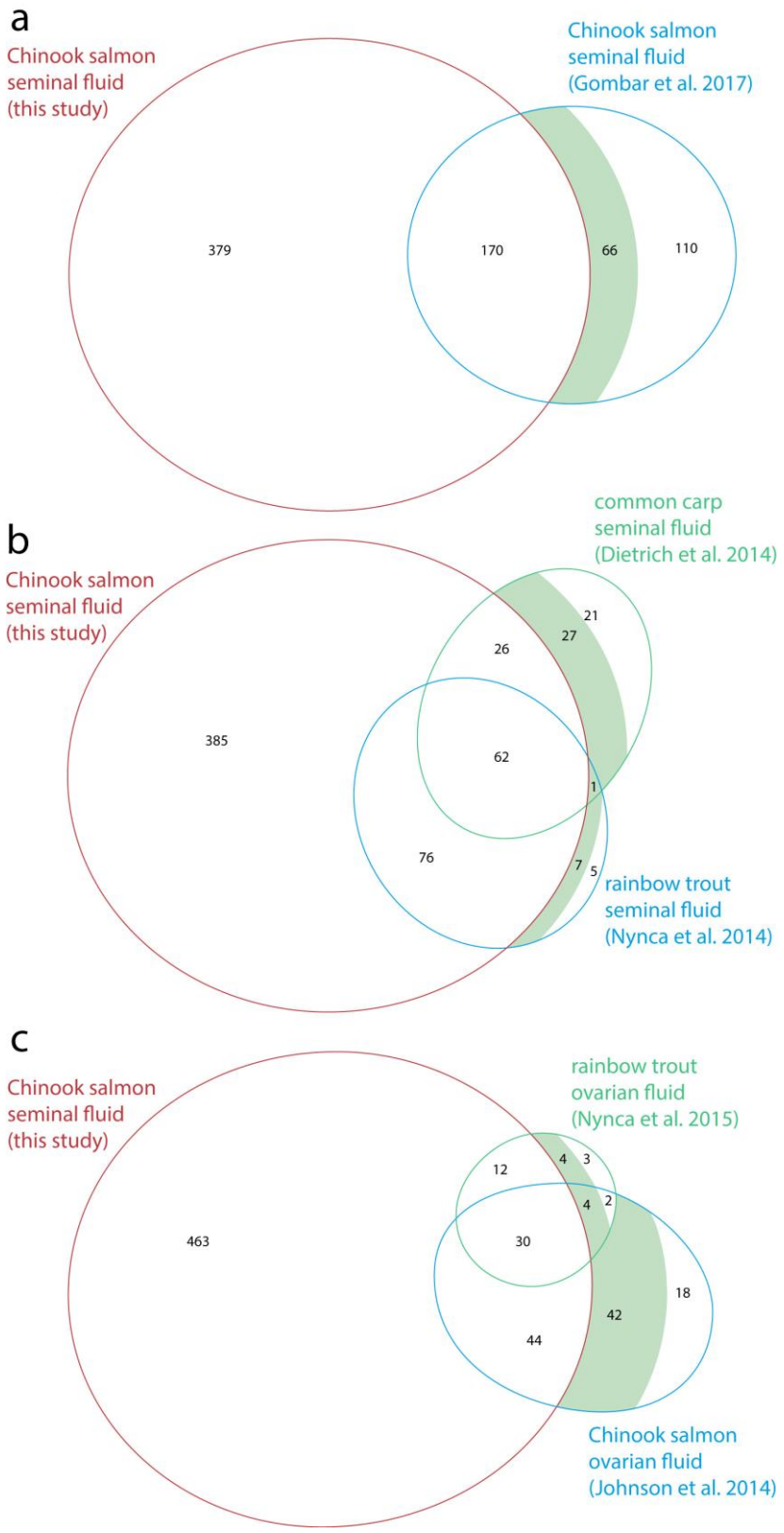

Figure 4.3: Comparison of chinook salmon seminal fluid proteins detected in this study to proteins identified previously in a) the seminal fluid of chinook salmon (Gombar et al. 2017), and b) common carp (Dietrich et al. 2014a) and rainbow trout (Nynca et al. 2014). Shaded areas show proteins not matching one of the 549 proteins from this study (based on sequence similarity) but identified by MASCOT when searching the MS/MS data from this study against that list. Diagrams generated using EulerAPE (Micallef and Rodgers 2014).

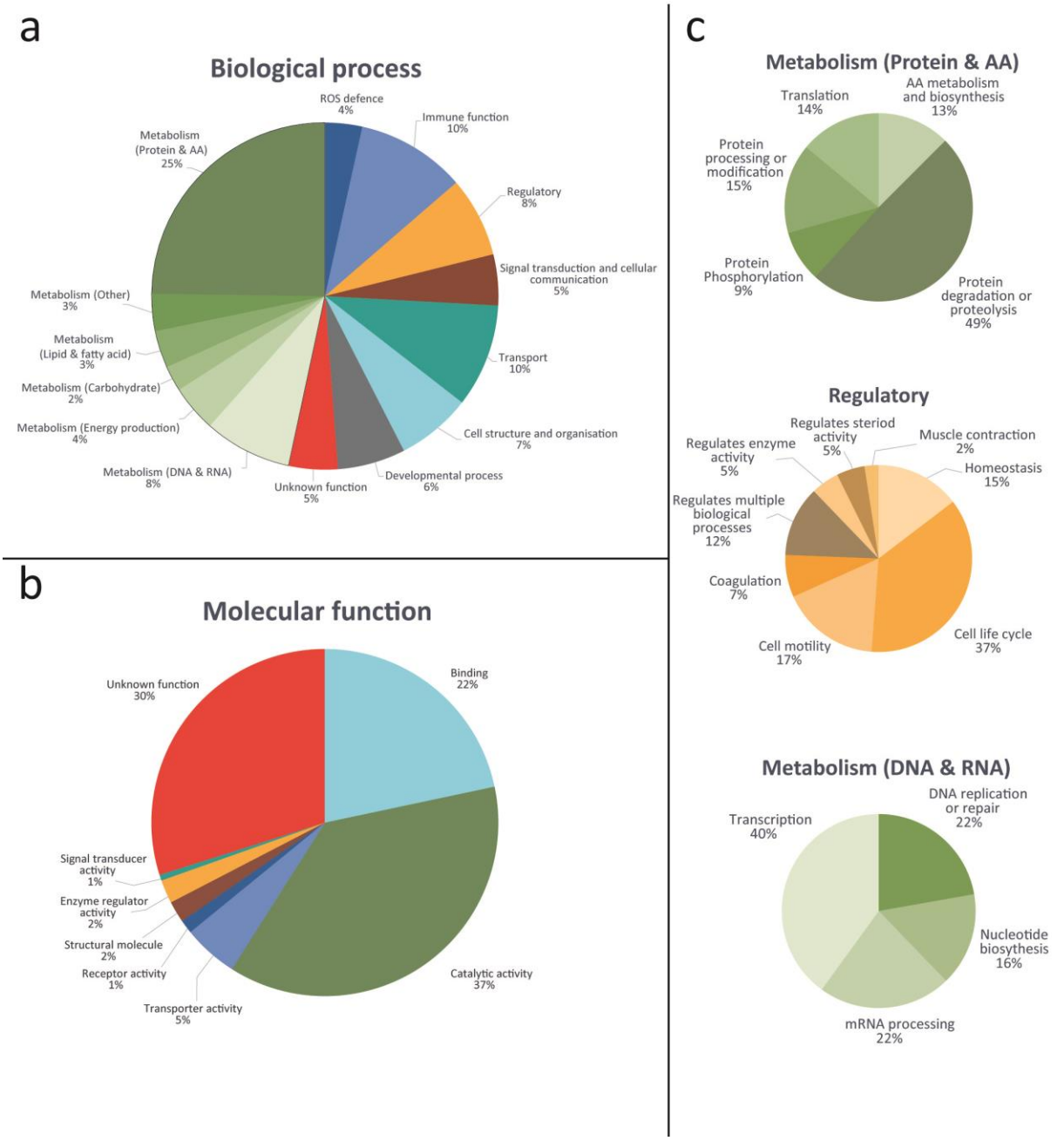

Figure 4.4: The gene ontology classification of chinook salmon seminal fluid proteins; a) broad "biological process", b) "molecular function", and c) detailed "biological process" for three of the broad categories.

Table 4.1: Proteins detected in Chinook salmon seminal fluid with biological functions that can potentially influence sperm motility. ROS = reactive oxygen species

Accession	Protein name	Biological function	Source
C0H9U3	6-phosphogluconate dehydrogenase, decarboxylating	Energy metabolism	UniProtKB
B9EPN0	6-phosphogluconolactonase	Energy metabolism	UniProtKB
B5DGM5	Adenylate kinase	Energy metabolism	UniProtKB
B5X9C6	Aldose reductase	Energy metabolism	UniProtKB
B5X9Z8	ATPase inhibitor, mitochondrial	Energy metabolism	UniProtKB
B5DGQ7	Beta-enolase	Energy metabolism	UniProtKB
P24722	Creatine kinase, testis isozyme	Energy metabolism	UniProtKB; (Wallimann et al. 2011)
B9EMZ7	Cytochrome c	Energy metabolism	UniProtKB
B5X0T0	Fructose-bisphosphate aldolase	Energy metabolism	UniProtKB
C0H9I1	Fructose-bisphosphate aldolase	Energy metabolism	UniProtKB
B5X1I3	Glucose-6-phosphate 1-dehydrogenase	Energy metabolism	UniProtKB
C0H9M4	Glucose-6-phosphate isomerase	Energy metabolism	UniProtKB
B5X3K2	Glyceraldehyde-3-phosphate dehydrogenase	Energy metabolism	UniProtKB
Q98SJ9	Glycerol-3-phosphate dehydrogenase	Energy metabolism	UniProtKB
B5X115	Glycogenin-1	Energy metabolism	(Hirohashi et al. 2016)
B5DGS3	Isocitrate dehydrogenase [NADP]	Energy metabolism	UniProtKB
C0HAI2	L-lactate dehydrogenase	Energy metabolism	UniProtKB
B5X5V8	L-xylulose reductase	Energy metabolism	UniProtKB
B5DFT8	Malate dehydrogenase	Energy metabolism	UniProtKB
B5XBK0	Malate dehydrogenase	Energy metabolism	UniProtKB
B5DG72	Phosphoglucomutase 1	Energy metabolism	UniProtKB
B5DFX8	Phosphoglycerate kinase	Energy metabolism	UniProtKB
B5DGT9	Phosphoglycerate mutase 2-1 (Muscle)	Energy metabolism	UniProtKB
C0PUK9	Pyruvate kinase	Energy metabolism	UniProtKB
B9EM17	Transaldolase	Energy metabolism	UniProtKB
B5DGL3	Triosephosphate isomerase	Energy metabolism	UniProtKB

C1BF07	Calmodulin	Cell motility	U3KED5_FICAL (100%); (Tash et al. 1988)
B5X3I8	Carbonic anhydrase	Cell motility	UniProtKB; (Inaba et al. 2003, Wandernoth et al. 2010, 2015, José et al. 2015)
Q6R4A2	Cytoplasmic carbonic anhydrase	Cell motility	(Inaba et al. 2003, Wandernoth et al. 2010, 2015, José et al. 2015)
C0PU84	Ectonucleotide pyrophosphatase/phosphodiesterase family member 2	Cell motility	UniProtKB; (Goding et al. 2003)
C0HAT9	Parvalbumin alpha	Cell motility	(Kagi et al. 1987, Dietrich et al. 2010, 2014b)
Q8AYB4	Parvalbumin beta 542	Cell motility	(Kagi et al. 1987, Dietrich et al. 2010, 2014b)
E1UJ20	Parvalbumin beta-1	Cell motility	(Kagi et al. 1987, Dietrich et al. 2010, 2014b)
C1BEZ4	Glutathione S-transferase A	ROS defence	(Strange et al. 2000, Hemachand and Shaha 2003, Aydemir et al. 2007)
B5XBZ2	Glutathione S-transferase P	ROS defence	(Strange et al. 2000, Hemachand and Shaha 2003, Aydemir et al. 2007)
P79825	Hemopexin-like protein	ROS defence	UniProtKB; (Tolosano and Altruda 2002)
Q9DFF1	Hemopexin-like protein variant 1	ROS defence	HEMO_DANRE (61%); (Tolosano and Altruda 2002)
Q0H908	Metallothionein	ROS defence	(Suzuki et al. 1994, Fabrik et al. 2008)

B5XBY3	Peroxiredoxin-1	ROS defence	UniProtKB
B5X5Q6	Peroxiredoxin-5, mitochondrial	ROS defence	UniProtKB
C1BHF2	Peroxiredoxin-6	ROS defence	UniProtKB
L7Z8X5	Protein disulfide-isomerase	ROS defence	UniProtKB
Q03156	Serum albumin 2	ROS defence	Uniprot GO; (Halliwell 1988, Roche et al. 2008)
B5X4I3	SH3 domain-binding glutamic acid-rich-like protein 3	ROS defence	UniProtKB
B5X6G0	SH3 domain-binding glutamic acid-rich-like protein 3	ROS defence	UniProtKB
Q8QHI0	Superoxide dismutase [Cu-Zn]	ROS defence	UniProtKB; (Celino et al. 2011)
C1BFF6	Thioredoxin	ROS defence	UniProtKB; (Arnér and Holmgren 2000, Pacitti et al. 2014)
B9ELD9	Thioredoxin domain-containing protein 12	ROS defence	UniProtKB; (Arnér and Holmgren 2000, Pacitti et al. 2014)
B5XAW4	Thioredoxin domain-containing protein 17	ROS defence	TXD17_EPICO(88%); (Arnér and Holmgren 2000, Pacitti et al. 2014)
B5XFI4	Thioredoxin-dependent peroxide reductase, mitochondrial	ROS defence	UniProtKB; (Arnér and Holmgren 2000, Pacitti et al. 2014)
B5X9C1	14 kDa phosphohistidine phosphatase	Protein Phosphorylation	F1Q626_DANRE (75%), (Shenolikar 1994, Wera and Hemmings 1995)
C0PUT9	Calcium/calmodulin-dependent protein kinase type II delta chain (Fragment)	Protein Phosphorylation	UniProtKB
C0HBG9	cAMP-dependent protein kinase catalytic subunit beta	Protein Phosphorylation	UniProtKB
B9EQ35	Coiled-coil domain-containing protein 75	Protein Phosphorylation	Q4RPZ3_TETNG (74%)
Q09IZ1	Cyclin B2	Protein Phosphorylation	UniProtKB

B5X7L5	Low molecular weight phosphotyrosine protein phosphatase	Protein Phosphorylation	UniProtKB
B5X590	Nicotinamide riboside kinase 2	Protein Phosphorylation	UniProtKB
B5X2R9	Nuclear ubiquitous casein and cyclin-dependent kinases substrate	Protein Phosphorylation	UniProtKB
B5X1L0	Serine/threonine-protein kinase Sgk2	Protein Phosphorylation	UniProtKB; (Shenolikar 1994, Wera and Hemmings 1995)
C0H8Y4	Serine/threonine-protein kinase VRK3	Protein Phosphorylation	UniProtKB; (Shenolikar 1994, Wera and Hemmings 1995)
B5X1D2	Serine/threonine-protein phosphatase	Protein Phosphorylation	UniProtKB; (Shenolikar 1994, Wera and Hemmings 1995)
B5X2G2	Serine/threonine-protein phosphatase	Protein Phosphorylation	UniProtKB; (Shenolikar 1994, Wera and Hemmings 1995)

4.2.5 DISCUSSION

We improve upon the previously published seminal fluid proteome in Chinook salmon and to our knowledge present the largest seminal fluid proteome to date for teleost fish. Our findings suggest initial studies that characterised the seminal fluid proteomes of rainbow trout (Nynca et al. 2014) and common carp (Dietrich et al. 2014a) were able to determine the core subset of SFPs that appear to be well conserved across species (Figure 4.3b). There is less overlap between our proteome list and the recent list published for the same species (Figure 4.3a), this is somewhat surprising and possible explanations include differences in the treatment of seminal fluid prior to MS analysis or that Gombar et al. (2017) sampled males with different life-histories where we sampled males with the same life-history that varied in social status. Although there are differences in the methods used, both this study and the recent work (Gombar et al. 2017) on Chinook salmon highlight that gel free proteomic methods can improve the number of proteins detected compared to the use of gel prefractionation methods alone. Using GO analysis, we were able to highlight functional groups of interest

with respect to sperm motility and therefore proteins that possibly impact the outcome of sperm competition (Table 4.1).

Motility of fish spermatozoa results in the rapid consumption of stored energy sources within the cell and the high energy demands of flagellar movement quickly outstrip energy production rates resulting in the cessation of movement (Dzyuba et al. 2017). Furthermore, sperm velocity is closely linked to flagellar beat frequency, which is proportional to dynein-ATPase activity (Cosson et al. 2008, Dzyuba et al. 2017), and sperm ATP levels have been positively correlated with sperm velocity (Lahnsteiner et al. 1998, Bencic et al. 1999, Burness et al. 2004) and fertilisation success (Zilli et al. 2004, Vladić et al. 2010) in external fertilisers. The major source of energy production in sperm prior to activation of motility is oxidative phosphorylation that maintains a store of ATP ready for use when motility is triggered (Dzyuba et al. 2017). In the short term following activation of motility in salmonids, sperm utilise ATP as the energy source for flagellar movement (Christen et al. 1987) using both stored ATP reserves and increasing ATP production significantly via aerobic respiration (Lahnsteiner et al. 1993a, 1999). We found 25 proteins involved in energy metabolism pathways in Chinook salmon seminal fluid (Table 4.1). These include the enzymes Adenylate kinase (AK) and Creatine kinase (CK), that work to regenerate ATP levels using ADP and creatine-phosphate as substrates within flagella (Dzyuba et al. 2016, 2017). Additionally, AK may influence the ratio of ATP/ADP which also regulates dynein activity (Yoshimura et al. 2007). Other proteins detected include Pyruvate kinase, Lactate dehydrogenase and Malate dehydrogenase, which along with AK have activity levels previously correlated with fish sperm motility (Lahnsteiner et al. 1996, 1998).

Another function of interest is the protection of sperm from reactive oxygen species (ROS) for which we detected 18 proteins (Table 4.1). The high energy demands of sperm motility are directly linked to ROS generation, as a major source of ROS generation in sperm is mitochondrial energy production (Aitken and Curry 2011). In external fertilisers, sperm released into the water may also face oxidative stress from environmental xenobiotics (Dzyuba et al. 2017). ROS can damage sperm DNA and attack membranes via lipid peroxidation (de Lamirande 1997, Aitken and Curry 2011, Gao et al. 2017), this damage results in significant negative effects on fish sperm motility and velocity (Gazo et al. 2013, Hulak et al. 2013, Linhartova et al. 2013, Shaliutina et al. 2017). Antioxidants in seminal fluid can

protect sperm from oxidative damage and enzymes with antioxidant properties in fish seminal fluid have a positive effect on sperm velocity (Mansour et al. 2006, Lahnsteiner et al. 2010, Lahnsteiner and Mansour 2010). In addition to damaging sperm, ROS could alter sperm velocity by interfering with signalling pathways that regulate motility, in particular by negatively regulating kinases and phosphatases involved in signalling pathways that regulate energy production or dynein activity (Aitken and Curry 2011, Dzyuba et al. 2017, Zilli et al. 2017).

Interlinked Ca^{2+} mediated signalling pathways are involved in the regulation of fish sperm motility. In salmonids, sperm motility is initiated upon exposure of sperm to the external environment that results in a decrease in external K^+, triggering efflux of K^+ from sperm and influx of Ca^{2+} via membrane ion channels (Alavi and Cosson 2006, Zilli et al. 2017). This influx of Ca^{2+} and the calcium binding protein Calmodulin (CaM) create a change in membrane potential that stimulates cAMP production via adenylyl cyclase and sets in motion the cAMP-dependent phosphorylation of several proteins initiating sperm motility (Morisawa and Okuno 1982, Inaba et al. 1998, Itoh et al. 2001, Kho et al. 2004, Morisawa and Yoshida 2005, Alavi and Cosson 2006, Morisawa 2008, Zilli et al. 2017). Ca^{2+}/CaM or cAMP dependent phosphorylation may also regulate dynein activity during motility (Porter and Sale 2000, Morita et al. 2006, Dymek and Smith 2007). External Ca^{2+} levels can also influence sperm motility; greater sperm velocity is observed for sperm swimming in the presence of Ca^{2+} in the European perch (*Perca fluviatilis*) (Alavi et al. 2007) and the European eel (*Anguilla anguilla*) (Pérez et al. 2016). We detected several proteins in Chinook salmon seminal fluid including CaM and Ca^{2+} binding Parvalbumin proteins (Table 4.1) that may influence Ca^{2+} signalling.

Protein phosphorylation is a key part of the pathway that initiates sperm motility and we detected 12 protein kinases/phosphatases (Table 4.1). In salmonids, cAMP-dependent phosphorylation of at least 7 proteins initiates sperm motility, including a light chain of the outer arm dynein and regulatory subunit of protein kinase-A (Itoh et al. 2001). Motility of sperm is controlled by the activity of dynein, the molecular motor that powers coordinated sliding of microtubules in the axoneme and therefore flagellar movement (Dumorné et al. 2017, Dzyuba et al. 2017, Zilli et al. 2017). As such, processes and pathways influencing dynein activity are likely to alter cell motility, and phosphorylation state of dynein proteins is an

important aspect of local control over dynein motor activity (Porter and Sale 2000, Morita et al. 2006, Dymek and Smith 2007). Relatively little is known about the role that protein phosphorylation plays in regulating dynein activity *during* fish sperm motility, however in addition to changes directly to the phosphorylation state of dynein proteins, phosphorylation of signalling proteins and proteins in energy metabolism pathways could all potentially influence sperm velocity (Zilli et al. 2017).

We found 67 proteases/protease inhibitors in Chinook salmon seminal fluid, representing 12% of the total proteome, and find 35 proteases/protease inhibitors not previously identified in teleost seminal fluid (Supplement 1: full protein list). Proteases and their inhibitors feature as a major functional group of SFPs (Mueller et al. 2004, LaFlamme and Wolfner 2013), and have been found in the seminal fluid of vertebrate species including humans (Pilch and Mann 2006), several ruminants (Druart et al. 2013) and chickens (Labas et al. 2015), and invertebrates including *Drosophila melanogaster* (Findlay et al. 2008, Takemori and Yamamoto 2009), honey bees (*Apis mellifera*) (Baer et al. 2009, Grassl et al. 2017), mosquitos (*Aedes aegypti* and *Ae. albopictus*) (Sirot et al. 2011, Boes et al. 2014) and a seed beetle (*Callosobruchus maculatus*) (Bayram et al. 2017). These enzymes play key roles in the regulation of several reproductive processes including semen coagulation, eliciting post-mating responses in females, immune response within the reproductive tract, sperm maturation and activation of sperm motility (Smith and Stanfield 2011, LaFlamme et al. 2012, Zhao et al. 2012, LaFlamme and Wolfner 2013, Dietrich et al. 2017). Furthermore, changes to SFP composition in rapid response to changing sperm competition risk would require a relatively rapid turnover of specific proteins, thus proteases and their inhibitors are likely potential regulators of such responses (LaFlamme and Wolfner 2013). The expression of proteases/protease inhibitors in seminal fluid across a wide range of taxa suggests at least some conserved function. It may be possible to elucidate some of these conserved functions by establishing the function of these proteins in external fertilisers where there is no opportunity for direct interaction and influence on female behaviour or physiology, although seminal fluid may interact with ovarian fluid.

As ovarian fluid can influence sperm velocity in salmonids (Dietrich et al. 2008, Rosengrave et al. 2008, 2009, 2016), comparing the proteome of ovarian and seminal fluids may help to identify the unknown mechanism involved in sperm-fluid interaction. The significant overlap

observed between ovarian and seminal fluids likely reflects the similar role of storage and protection of gametes, and possibly reflects a similar mechanism of interaction with sperm. Johnson et al. (2014) note that Chinook salmon ovarian fluid protein composition appears to vary among females and propose several proteins that may influence sperm velocity, including Complement proteins and the aspartic protease cathepsin D, both of which we have detected in seminal fluid. The high degree of similarity between seminal and ovarian fluid protein lists may also be a product of the relatively low number of proteins detected in ovarian fluid thus far and highlights the need for improved characterisation of the ovarian fluid proteome in externally fertilising fishes.

While many of the proteins discussed have functions described within sperm cells and their extracellular function is uncertain, it is possible that these "cellular" proteins are detected in seminal fluid because they are in the process of being shuttled to sperm within exosomes. Exosomes, or extracellular vesicles, excreted by cells into body fluids including seminal fluid, are potentially linked to sperm performance as they play a role in signalling pathways, immune response and in regulating sperm motility (Raposo and Stoorvogel 2013, Aalberts et al. 2013, Vojtech et al. 2014). Gombar et al. (2017) also detected proteins in Chinook salmon seminal fluid that could be classed as exosomal cargo, and indeed provide some additional support for the idea that exosomes may shuttle important proteins to salmon sperm, as they found that Lactose dehydrogenase, an energy metabolism protein found in higher abundance in jack male seminal fluid, also occurred in higher levels within isolated seminal fluid exosomes from jacks. In mammals, exosomes are important mediators of the sperm maturation process that occurs in the epididymis, as they shuttle various molecules from the epithelial cells lining the epididymis to maturing spermatozoa (Saez et al. 2003, Sullivan et al. 2005, 2007, Rowlison et al. 2018, Zhou et al. 2018). While fish lack an epididymis, some kind of maturation process is thought to occur in salmonids because sperm taken from the testis before passage through the spermatic duct do not achieve motility when entering water (Schulz et al. 2010). Salmonid testis lack the accessory glands (Chowdhury and Joy 2007) that are associated with secreted SFPs in other organisms, and secretions into seminal fluid occur via the epithelium of the spermatic duct (Lahnsteiner et al. 1993b), however the role that exosomes play within salmonid testis has not been characterised and warrants further investigation.

In conclusion, implementing a combination of prefractionation techniques followed by LC-MS/MS has allowed us to improve the characterisation of the chinook salmon seminal fluid proteome. These results progress our understanding of the SFP composition of teleost species and aid in gaining a better understanding of the role that SFPs may play in sperm competition for externally fertilising fish.

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CHAPTER FIVE

IDENTIFYING CANDIDATE PROTEINS INVOLVED IN SPERM AND SEMINAL FLUID INTERACTION IN CHINOOK SALMON

5.1 PREFACE

This chapter consists of analyses and results for a manuscript in preparation for submission to a Special Issue in Molecular & Cellular Proteomics. The final manuscript will combine results from this chapter with the descriptive proteomic analyses from Chapter Four.

The proteome refers to the entire protein complement expressed by a genome in a given cell, tissue or organism at a given time (Wilkins et al. 1996). Proteomics is the application of various methods that attempt to describe all of these proteins (Findlay and Swanson 2010, Diz et al. 2012, Valcu and Kempenaers 2014, Baer and Millar 2016). The use of proteomic methods to characterise differential expression of proteins has applications in ecology, behaviour, reproduction and evolution (Findlay and Swanson 2010, Diz et al. 2012, Valcu and Kempenaers 2014, Baer and Millar 2016). For example, proteomic methods can aid in determining the biochemical and physiological basis of an organism's response to selection, which is critical to further our understanding of adaptive evolution (Diz et al. 2012, Baer and Millar 2016). While proteomic methods are useful tools that can be used to establish the molecular basis of phenotype, in turn studies utilising behavioural manipulations can reveal much about the response of proteomes to the social environment (Sirot 2015).

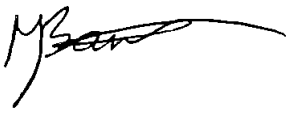
In Chapter Two, by manipulating social status and therefore sperm competition risk, I demonstrated that male Chinook salmon rapidly respond to a change from dominant to subdominant status by increasing the velocity of their sperm. By manipulating ejaculates in Chapters Two and Three, separating and recombining sperm and seminal fluid from different individuals, I have shown that males with greater sperm competition risk invest in high quality seminal fluid that has a significant influence on sperm velocity.

The major aim in this chapter was to identify seminal fluid proteins that may be involved in the proximate mechanism behind the sperm and seminal fluid interactions described in previous chapters. Here, I examine differential expression of the seminal fluid proteome among males of different social status (dominant and subdominant), using seminal fluid samples collected during the 2014 field season as I conducted the social manipulation experiment reported in Chapter Two. Using this subset of males, I found no significant association between sperm velocity and social status which meant that I was unable to test the prediction that the relative abundance of proteins in seminal fluid with functions that may

influence sperm motility (as outlined in Chapter Four) will differ between dominant and subdominant males. However, because sperm velocity varied among this subset of males, I was able to test for correlation between protein abundance and sperm velocity. Ultimately, this research provides a basis for future investigations that can use the information presented here to guide the development of hypotheses and more targeted experiments that investigate sperm and seminal fluid interaction in externally fertilising fish and beyond.

5.1.1 STATEMENT OF CONTRIBUTION

As lead author of the following manuscript in preparation, I conducted all laboratory work while under supervision of Dr Julia Grassl and Prof. Boris Baer. Dr Patrice Rosengrave and myself conducted all field work. I performed all analyses of the data, including processing of quantitative proteomic data in Skyline and statistical analyses. I wrote the first and final drafts and designed all figures and tables. I was provided with comments, edits and input from all contributing authors.



Michael J. Bartlett

5.2 LINKING VARIATION IN THE SEMINAL FLUID PROTEOME WITH EJACULATE QUALITY TRAITS IN CHINOOK SALMON (*ONCORHYNCHUS TSHAWYTSCHA*)

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5.2.1 ABSTRACT

If females mate with multiple males, the ejaculates of those males compete for egg fertilisation resulting in sperm competition. Males often increase total sperm number per ejaculate to outcompete their rivals, but they are also able to alter sperm performance, for example by increasing sperm velocity. There is increasing evidence that seminal fluid, in particular seminal fluid proteins (SFPs), are key drivers of sperm competition and reproductive success, but their identity and functioning is still poorly understood. As already pointed out in Chapter Two, seminal fluid mediates rapid changes to sperm velocity in Chinook salmon (*Oncorhynchus tshawytscha*). In this species, males respond to changes in social status and increase velocity of sperm with increased sperm competition risk. Here, our intent was to continue unravelling the underlaying molecular mechanisms by testing the prediction that the relative abundance of proteins in seminal fluid with functions that may influence sperm motility (as outlined in Chapter Four) will differ between dominant and subdominant males. However, because only a subset of males from the experiment in Chapter Two were available for proteomic analysis, and there was no significant relationship between either sperm velocity or number and social status for these males, we were unable to test this prediction. Instead, we focused on relationships between protein abundance and sperm velocity and found significant correlations between 36 proteins and ejaculate quality traits. We also detected 26 proteins which differed significantly between dominant and subdominant males. Several of these proteins have biological functions that are relevant to sperm physiology and provide promising avenues for future research investigating the proximate mechanism behind sperm and seminal fluid interactions in salmonids. While much remains to be discovered, our results provide important initial insight into the pivotal role that seminal fluid proteins play in strategic investment in ejaculate.

5.2.2 INTRODUCTION

Polyandry, or multiple mating by females, is a widespread phenomenon (Zeh and Zeh 2003, Simmons 2005, Parker and Birkhead 2013, Taylor et al. 2014) resulting in competition among ejaculates from rival males for the fertilisation of ova (Parker 1970). Sperm competition theory predicts that if female remating probability is high (sperm competition risk), males should tailor their ejaculate investments to maximise paternity, both among species (Parker and Pizzari 2010) and among males within the same species (Wedell et al. 2002, Parker and Pizzari 2010). Supporting this, many studies have found that sperm competition can result in increased sperm production (reviewed by Simmons and Fitzpatrick 2012, Parker 2016) and allocate ejaculates containing more sperm in response to the presence of a rival (delBarco-Trillo 2011, Kelly and Jennions 2011). There is also accumulating evidence that apart from increased expenditure in sperm numbers, males also alter key "sperm quality" traits such as velocity or viability (Snook 2005, Simmons and Fitzpatrick 2012, Fitzpatrick and Lüpold 2014). For example, in externally fertilising fish species, males with faster swimming sperm fertilise a higher proportion of eggs relative to a competitor (Gage et al. 2004, Liljedal et al. 2008, Evans et al. 2013, Egeland et al. 2015, Rosengrave et al. 2016). A number of studies now provide evidence that sperm quality traits are influenced by the non-sperm component of an ejaculate or seminal fluid (Poiani 2006, Cameron et al. 2007, Alonzo and Pizzari 2010, Simmons and Fitzpatrick 2012, Perry et al. 2013, Dhole and Servedio 2014, Fitzpatrick and Lüpold 2014). However, the components of seminal fluid that are responsible for such effects on sperm performance in many cases are unknown.

Seminal fluid constituents, in particular seminal fluid proteins (SFPs), are indeed known to be key determinants of several sperm quality traits, influencing sperm survival and fertilisation ability (den Boer et al. 2008, Holman 2009, den Boer et al. 2010, King et al. 2011, Rodríguez-Martínez et al. 2011, Simmons and Beveridge 2011, Mendoza et al. 2013, Rodrigues et al. 2013). SFPs can also influence female behaviour and physiology and thereby impact fitness of both sexes (Chapman and Davies 2004, Robertson 2005, Chapman 2008, Avila et al. 2011, Schjenken and Robertson 2014, Sirot et al. 2015). Therefore, considering both sperm and seminal fluid ("ejaculate quality") as opposed to focusing on sperm number alone is important to understand the evolution of ejaculate investment strategies (Poiani 2006, Cameron et al. 2007, Alonzo and Pizzari 2010, Perry et al. 2013, Dhole and Servedio 2014).

There is increasing evidence that males can rapidly alter sperm and/or ejaculate characteristics in response to perceived and real changes in sperm competition risk (Zbinden et al. 2003, 2004, Kilgallon and Simmons 2005, Bretman et al. 2009, 2010, 2012, Smith and Ryan 2011, Moatt et al. 2014, Fitzpatrick and Lüpold 2014, Burger et al. 2015). However, in most of these cases the underlying molecular mechanisms have not been unravelled in any great detail. For example, adjustment of sperm motility in response to perceived sperm competition risk has been reported for humans (Kilgallon and Simmons 2005), horses (*Equus caballus*) (Burger et al. 2015), and the swordtail fish (*Xiphophorus nigrensis*) (Smith and Ryan 2011). How these species make rapid changes to sperm performance is unknown and the role of seminal fluid plays in these changes in sperm phenotype remains to be determined.

Recent proteomic and transcriptomic approaches have revealed a number of candidate proteins involved in strategic ejaculate investments. In house mice (*Mus domesticus*) (Ramm et al. 2015), and field crickets (*Teleogryllus oceanicus*) (Simmons and Lovegrove 2017, Sloan et al. 2018) differential expression of subsets of seminal fluid proteins was correlated with sperm competition risk or intensity. There is also evidence that SFPs may mediate the complex strategic adjustment of ejaculate quality made by fowl (*Gallus gallus*). Male fowl strategically adjust sperm velocity, which is a key trait linked to competitive fertilisation success (Birkhead et al. 1999, Pizzari et al. 2008), based upon their social status, future mating opportunities and perceived female quality (Pizzari et al. 2007, Cornwallis and Birkhead 2007, Cornwallis and O'Connor 2009). Fowl produce higher quality ejaculates as they get older, and a recent proteomic study of seminal fluid compared males with different ages finding that the abundance of nine SFPs was associated with increased sperm velocity (Borziak et al. 2016). Combined, these recent studies suggest that SFPs influence sperm quality traits and are potential mediators of the rapid ejaculate adjustments males make in response to social cues.

In this research, we use Chinook salmon (*Oncorhynchus tshawytscha*), an externally fertilising fish with a dynamic social environment experiencing substantial variation in sperm competition risk (Esteve 2005). In this species, large "hooknose" males fight to establish social dominance and monopolise access to females, while subdominant males attempt to sneak fertilisations (Esteve 2005). Subdominant males that adopt a sneaking tactic have a greater risk of sperm competition because they always compete with dominant males, therefore in Chapter Two (Bartlett et al. 2017), we predicted that subdominant males will make greater

investment in ejaculate quality. To test this prediction, we manipulated male social status of hooknose males and found that they strategically invest in ejaculates, as subdominant males produced ejaculates with more and faster swimming sperm. We also found that males changing from dominant to subdominant status quickly adjusted ejaculate traits by producing faster swimming sperm within 48 hours of changing status. The use of *in-vitro* ejaculate manipulations and sperm competition trials furthermore demonstrated that rapid changes in sperm velocity were mediated by seminal fluid and that such changes in velocity influenced male reproductive success. This is consistent with previous research showing that sperm velocity in the primary determinant of fertilisation for Chinook salmon and other salmonids when sperm from different males compete (Gage et al. 2004, Evans et al. 2013, Egeland et al. 2015, Rosengrave et al. 2016)

Here, we used seminal fluid collected during the social status manipulation as described in Chapter Two, and quantified differences in the seminal fluid proteome. We predict that the relative abundance of proteins in seminal fluid with functions that may influence sperm motility (as outlined in Chapter Four) will differ between dominant and subdominant males. Little is known about the possible SFPs that may be linked to sperm velocity in externally fertilising fish, however we focus our search on SFPs < 50 kDa in size, as seminal fluid fractions containing unknown proteins of this size had a significant effect on sperm velocity in the closely related rainbow trout (*O. mykkis*) (Lahnsteiner et al. 2004, Lahnsteiner 2007). Using 1D-PAGE prefractionation followed by LC-MS/MS analysis we quantified protein abundance with a spectral counting method and looked for correlation between protein abundance with male social status, sperm velocity and sperm concentration.

5.2.3 MATERIALS AND METHODS

Study species and seminal fluid collection

Wild chinook salmon (*Oncorhynchus tshawytscha*) were caught during their annual spawning run in a trap located on the Kaiapoi River, a tributary of the Waimakariri River system, Canterbury, New Zealand (Unwin et al. 2000). We studied a total of 17 sexually mature 3-year-old “hooknose” males captured between 27 April and 30 May in 2014. Males were subject to a two-stage social status manipulation experiment in which they were paired and social status (dominant or subdominant) was assessed at both stages, after which milt was collected using abdominal massage (Chapter Two; Bartlett et al. 2017).

Seminal fluid samples for proteomic analysis were prepared from milt collected at both experimental stages (n = 17 males, a total of 34 samples) that was stored on ice within 10 minutes of collection. Milt was centrifuged at 4 °C, 300 x g for 20 minutes to separate sperm cells from seminal fluid. This initial slow speed was used to minimise damage to sperm cells and therefore release of cellular proteins into the seminal fluid. The seminal fluid was then centrifuged at 4 °C, 20 000 x g for 20 minutes and then immediately frozen at -80 °C prior to further experimentation.

Measurement of ejaculate quality traits

Both sperm velocity and sperm concentration were determined for each male after collection of milt in each experimental stage as described in Chapter Two (see Figure 2.1 for social manipulation experimental design). Briefly, we measured sperm velocity at 10 s post-activation using a CEROS sperm tracker (v1.2, Hamilton-Thorne Research, Beverly, MA, USA). We used average path velocity (VAP, μm s^{-1}) which estimates the average velocity of a sperm cell for 0.5 s over a smoothed path (Rosengrave et al. 2008, 2009, 2016) as our measure of sperm swimming speed. VAP is known to be correlated with reproductive success in chinook salmon (Evans et al. 2013, Rosengrave et al. 2016, Chapter Two; Bartlett et al. 2017). Sperm concentration (sperm/ml) was determined for each milt sample using an improved Neubauer haemocytometer.

Sample prefractionation and digestion

Protein concentration of each sample was determined using a Bradford assay and 30 μg of protein was then loaded onto 1D SDS-PAGE gels which were run at 200 V for 45 minutes. After staining with Coomassie Brilliant Blue G-250, the < 50 KDa section of each lane (Chapter Four: Figure 4.2a) was used for MS analysis. The gels were destained and dehydrated with acetonitrile, gel pieces were then allowed to swell in 50 μL of 1 ng/ μl trypsin (Promega) in 10mM ammonium bicarbonate, on ice for 30 minutes, before incubating at 37 °C overnight. Samples were resuspended in 50 μL of 5% HPLC acetonitrile/0.1% formic acid (v/v) before passing through 0.22-mm centrifugal filters (Millipore) to remove any gel pieces.

Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis

LC-MS/MS was performed with samples analysed in quadruplicate (8 μg per analysis) on an Agilent 6550 Q-TOF with Chip Cube interface and C18 trapping/analytical Polaris chip using

45 min of 10% to 30% (v/v) acetonitrile gradients in 0.1% (v/v) formic acid. Settings used were positive ion mode, eight mass spectrometry (MS) scans at 250 to 1,400 mass-to-charge ratio per second, maximum of eight precursors per cycle with an absolute threshold of 5,000, scan speed varied according to abundance, and charge state selection set to +2 and +3 and selected by abundance.

Measurement of relative protein abundance

Resultant spectra were searched against a combined Salmoninae and common contaminant database (17,639 and 58 sequences respectively) using Mascot 2.5.1 (Matrix Science), employing an exclusion list method (Eubel et al. 2008) resulting in the identification off 549 proteins with a minimum Mascot score of 27 (FDR 2%) (Chapter Four). The resultant *.dat files were imported into Skyline 3.7 (MacLean et al. 2010) and associated with their corresponding *.d folders. Peptides were associated with a list of the 371 seminal fluid proteins < 50 KDa in size detected in this study. MS1 full-scan features were associated with MS/MS identifications (Schilling et al. 2012), and were manually checked for appropriate integration (i.e., peptides that could not be reliably integrated were removed; for details see Appendix D). For 149 peptides, peaks could not be integrated in one sample and for 5 peptides peaks could not be integrated for two samples; these peptides were retained and for the samples in which they could not be scored were treated as missing data. Only proteins with two or more peptides were retained for analyses post filtering, in total 23 proteins were removed. The area under the curve of each of the 2355 peptides from the remaining 348 proteins (average number of peptides per protein = 7, range = 2 – 31) was exported.

Two of the MS runs produced chromatograms that could not be scored and were removed from further analyses. Thus, data for two males were analysed from a single sample collected after the first social challenge, and data for the remaining 15 males were from samples collected at both stages (Table 5.1).

Table 5.1: Samples with protein abundance data available for further analysis. Two of the MS runs failed to produce scorable chromatograms, both runs were for samples from subdominant males in the second stage that were dominant in stage 1.

Social status phenotype	Social status in stage 1 (n)	Social status in stage 2 (n)
Dominant – Dominant	Dominant = 5	Dominant = 5
Dominant – Subdominant	Dominant = 4	**Subdominant = 2**
Subdominant – Dominant	Subdominant = 4	Dominant = 4
Subdominant – Subdominant	Subdominant = 4	Subdominant = 4
Total each stage	17, D = 9, S = 8	15, D = 9, S = 6

Statistical analysis

Data analysis was conducted using R v3.4 (R Core Team 2017). Raw data were processed with median normalisation and $\log_2$ transformation before further analysis using the package "MSstats" (Choi et al. 2014). Three different generalised linear mixed-effects models (GLMM) were fit for each protein using the package "lme4" (Bates et al. 2015). Protein abundance was used as the response variable for all models. The first model was used to test for as association between protein abundance and social status for samples collected in stage 1 of the experiment (factor with two levels, dominant = 9, subdominant = 6), sperm velocity and sperm concentration and were included as fixed effects. The second model was used to test for an association between protein abundance and social status for samples collected in stage 2 of the experiment with the same model parameters above (factor with two levels, dominant = 9, subdominant = 6). A final model was fit using data for samples collected in both experimental stages with the same model parameters above. This final model additionally included experimental stage in which samples were collected as a covariate and because data were used for the same males collected at both stages, male identity was also included as a random predictor to account for repeated measures. As values for the fixed effects sperm velocity and sperm concentration used different units, both of these variables were scaled prior to analyses. Additionally, models included the week during the spawning season when milt samples were collected as a random predictor to control for potential seasonal effects on milt quality (Butts et al. 2010, Hajirezaee et al. 2010).

In addition, GLMMs were used to test for an association between social status and the ejaculate parameters, sperm velocity and sperm concentration in both experimental stages with week as a random predictor as described above.

To determine the significance of fixed effects, we present both 95% Confidence Intervals (CI) calculated using the Wald method, and P values calculated for linear mixed effects models with the package "lmerTest" (Kuznetsova et al. 2017) using Satterthwaite approximations to calculate degrees of freedom. An alpha value of 0.05 was used to evaluate the significance of P-values. Assumptions underlying parametric models were verified using residual plots and Shapiro tests. Refer to Appendix E: *Chapter Five: Statistical analysis and R code*, for all R code used and output from analyses.

5.2.4 RESULTS

Contrary to our findings in Chapter Two (Bartlett et al. 2017), we found no significant difference between either sperm velocity or sperm concentration and social status in either experimental stage (Table 5.2, Figures 5.1 and 5.2). However, the data used here is restricted to samples collected in 2014 only (i.e., the subset of data available for proteomic analyses). Furthermore, significant changes in sperm velocity reported in Chapter Two were mainly driven by the dominant to subdominant group, for which two MS runs failed to produce protein abundance data (Table 5.1).

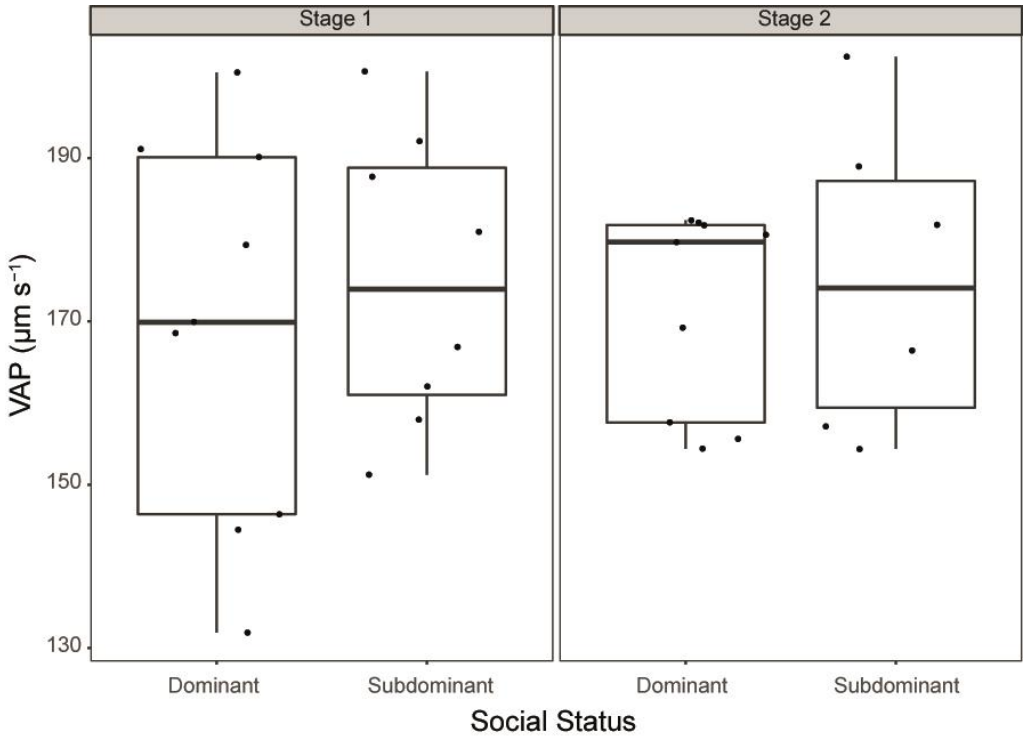

Figure 5.1: Sperm velocity (VAP in µm s^{-1}) for the subset of males available for proteomic analysis with dominant and subdominant social status in stage 1 (dominant, n = 9; subdominant, n = 8) and stage 2 (dominant, n = 9; subdominant, n = 6) of the social manipulation experiment. Boxplots display the median of each group with the 25th and 75th percentiles and whiskers extend to data within 1.5 x the inter-quartile range

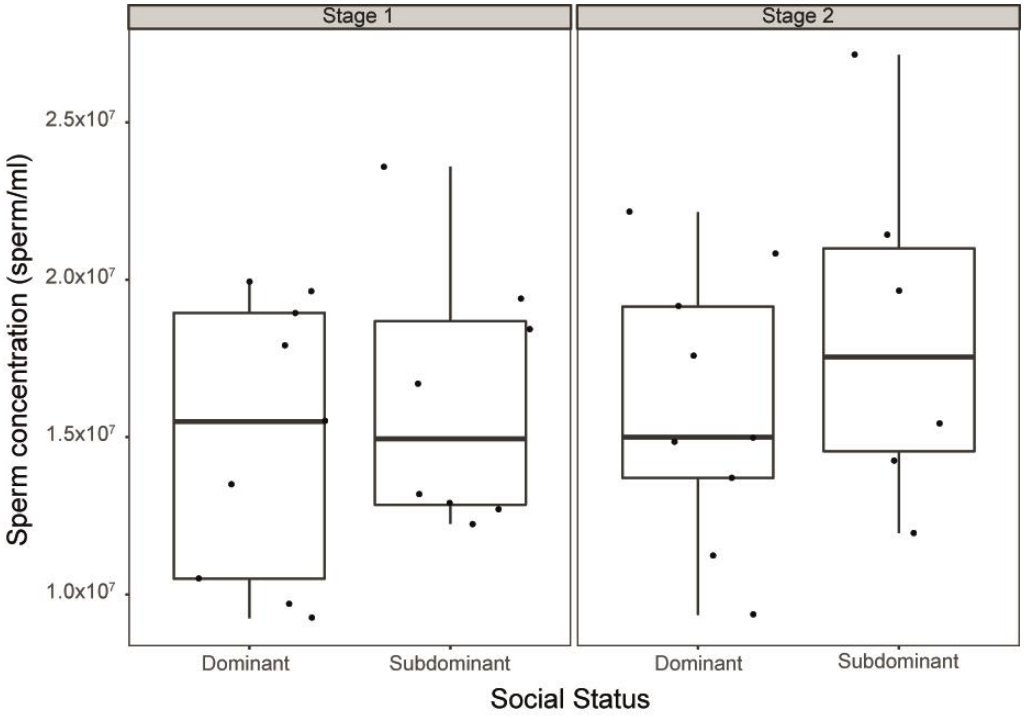

Figure 5.2: Sperm concentration (sperm/ml) for the subset of males available for proteomic analysis with dominant and subdominant social status in stage 1 (dominant, n = 9; subdominant, n = 8) and stage 2 (dominant, n = 9; subdominant, n = 6) of the social manipulation experiment. Boxplots display the median of each group with the 25th and 75th percentiles and whiskers extend to data within 1.5 x the inter-quartile range.

Table 5.2: Generalised linear mixed effects models (GLMM) to compare sperm velocity (VAP, μs^{-1}) and sperm concentration (cells/ml) in between males of dominant and subdominant social status in each experimental stage. *P*-values are calculated using Satterthwaite approximations to degrees of freedom and 95% Confidence Intervals were calculated using the Wald method.

Response variable	Experimental stage	Parameters (fixed effects)	estimate	95% CI	*P*-value
VAP	Stage 1	Intercept	168.8		
		Social status	5.4	-13.7 – 24.4	0.59
	Stage 2	Intercept	170.7		
		Social status	2.5	-8.7 – 13.7	0.67
Sperm concentration	Stage 1	Intercept	299.8		
		Social status	23.2	-57.8 – 104.3	0.58
	Stage 2	Intercept	317.1		
		Social status	46.9	-43.9 – 137.9	0.34

While the subset of males available for proteomic analysis from Chapter Two were not suitable to assess the link between social status and investment in ejaculate quality, we were still able to detect proteins that were differentially expressed between dominant and subdominant males. Moreover, although not associated with male social status, there was considerable variation in sperm velocity and sperm concentration among males (Figures 5.1 and 5.2). This allowed us to test for correlation between these ejaculate traits and protein abundance to determine whether proteins were associated with either sperm velocity of number.

Of the 348 proteins examined a total of 57 proteins (16 %) were associated with either male social status, sperm velocity and sperm concentration (Tables 5.S1-4). Of those, 38 proteins were classified as extra cellular based on TargetP-predicted secretory peptides or a description of extracellular localization in UniProtKB or previous research on reproductive fluids in teleost fishes (for methods see Chapter Four). Several functional groups of interest from these responsive proteins are composed mostly of secreted proteins, including all 11 proteins with immune function and all 6 proteins involved in proteolysis or its inhibition, as well as 9 of the 12 proteins with regulatory functions and 2 of the 3 proteins with anti-oxidant activity. Of the remaining 18 proteins not described as secreted, most were associated with either membranes or the cytoskeleton, and the remaining proteins are involved is several intracellular processes including transcription, mRNA splicing, intracellular signalling and transport (Tables 5.S1-4; Figure 5.3).

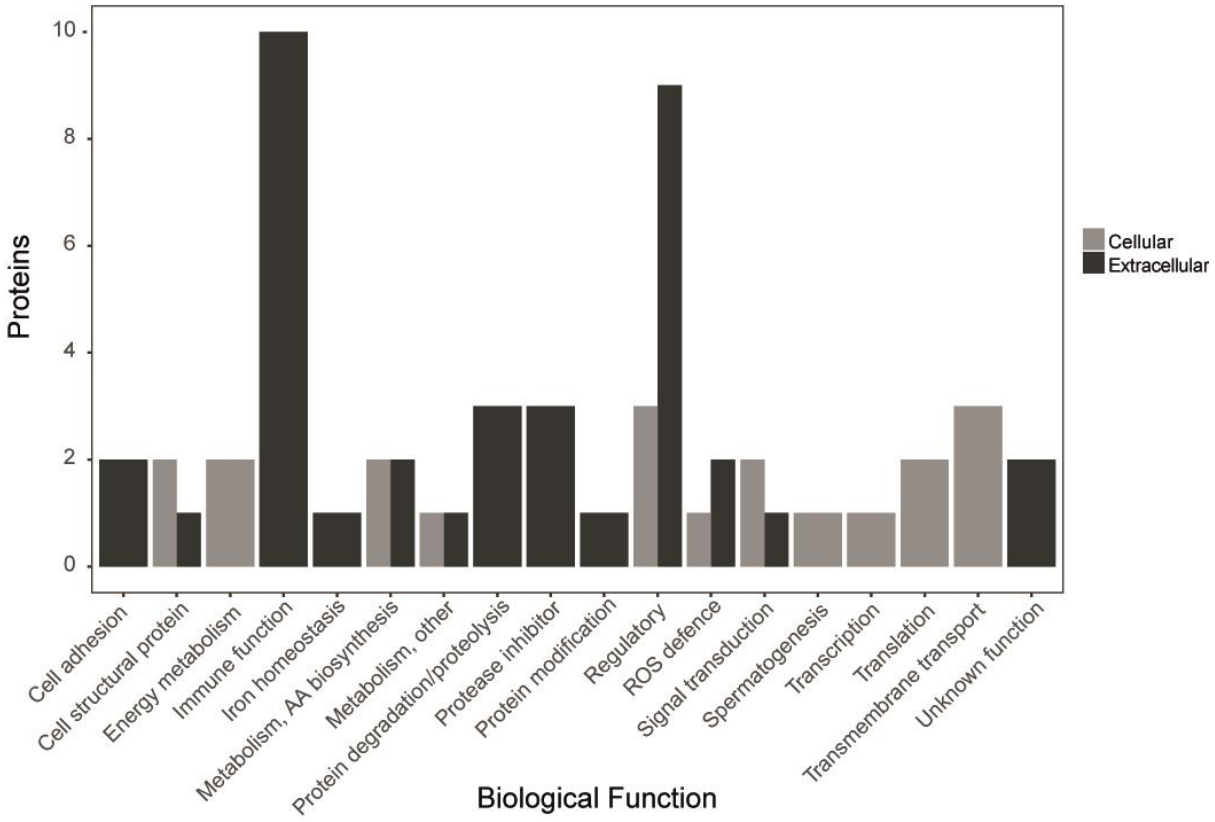

Figure 5.3: Summary of the extracellular localization for the 57 proteins that were associated with either male social status, sperm velocity and sperm concentration. Proteins are sorted by functional groups.

Comparing seminal fluid proteome between dominant and subdominant males revealed 26 proteins that differed significantly in abundance (Tables 5.S1 and 5.S2). Comparing males in the first stage of the experiment found 17 proteins were significantly associated with social status; abundance of 12 proteins was greater in dominant males, whereas 5 proteins were in higher abundance in subdominant males (Table 5.S1). Comparing males in the second stage of the experiment found only 5 proteins; abundance of 4 proteins greater in subdominant males (Table 5.S1). The analysis across all of the available data detected 17 proteins; abundance was significantly higher for 5 proteins in subdominant male's seminal fluid, whereas 12 proteins were significantly more abundant in dominant male's seminal fluid (Table 5.S2). Eight of the 12 proteins associated with dominant males and 2 of the 5 associated with subdominant males in stage 1 were also detected in the analysis across samples from both stages.

Altogether, we found that 36 proteins were significantly correlated with ejaculate quality measures. Of these, 8 proteins were positively correlated, and 10 proteins were negatively correlated with sperm velocity (Table 5.S3). We found that 10 proteins were positively correlated, and 10 were negatively correlated with sperm concentration (Table 5.S4). Two of these proteins were associated with both measures of ejaculate quality; abundance of both cytoplasmic carbonic anhydrase (CA2) and S-methyl-5'-thioadenosine phosphorylase (MTAP) showed a significant negative correlation with sperm velocity and sperm concentration.

5.2.5 DISCUSSION

Our comparison of the seminal fluid proteomes of dominant and subdominant males found that ejaculate quality of male Chinook salmon is linked to the protein composition of seminal fluid. This was indicated by patterns of differential protein abundance in 57 SFPs, of which 18 proteins were correlated with sperm velocity, 20 were correlated with sperm concentration and 28 proteins were associated with male social status. Overall, the gene ontology of these proteins varied across 16 functional groups, with the largest groups including 10 with immune function and 12 with regulatory functions, as well as 3 protease inhibitors. Furthermore, SFPs with functions linked to sperm motility, energy metabolism, and anti-oxidant activity were detected that warrant further discussion as these proteins are candidates for future research on the molecular interaction between sperm and seminal fluid in externally fertilising fish and other vertebrate species.

Seminal fluid proteome composition was linked to male social status, which appeared to be primarily driven by in the first stage of the experiment. However, we detected no significant relationship between measures of ejaculate quality and male social status that were detected in the full dataset (Chapter Two; Bartlett et al. 2017). The seminal fluid samples available for proteomic analysis were a subset of those from the experiment reported in Chapter Two. As such, the samples available for proteomic analysis were not ideally suited to testing the prediction, that seminal fluid protein abundance is linked to increased investment in ejaculate quality by subdominant males. Sperm velocity was variable among the males sampled in this study (Figure 1) and we were able to test for correlation between protein abundance and sperm velocity across all males, detecting several proteins of interest discussed below. Ultimately, sperm velocity is the key trait of interest for studies of sperm competition in salmonids and these results provide candidate seminal fluid proteins for future research.

Our results suggest that males may invest in increased antioxidant activity to protect sperm from oxidative attack and increase ejaculate quality. ROS can damage sperm DNA and cell membranes and could also influence signalling pathways that regulate motility (de Lamirande 1997, Winterbourn and Hampton 2008, Aitken and Curry 2011, Dzyuba et al. 2017, Zilli et al. 2017, Gao et al. 2017). The presence of ROS results in significant negative effects on fish sperm motility and velocity (Dietrich et al. 2005, Gazo et al. 2013, Hulak et al. 2013, Linhartova et al. 2013, Shaliutina et al. 2017) and antioxidant activity in seminal fluid of several fish species has a positive effect on sperm velocity (Lahnsteiner et al. 2010, Lahnsteiner and Mansour 2010). Similarly, house sparrows (*Passer domesticus*) alter their ejaculate quality in response to a change in male social status by increasing investments in ROS defence in response to sperm competition risk (Rojas Mora et al. 2017). In our analyses we found that Peroxiredoxin-1 (PRDX1) was found in higher abundance in ejaculates with faster swimming sperm (Table 5.S3). Peroxiredoxins reduce damaging H_2O_2 and mediate ROS signalling (O'Flaherty and Rico de Souza 2011, O'Flaherty 2014). A recent study in mice found that inhibition of PRDX resulted in a significant reduction of sperm velocity and intracellular ATP content (Ryu et al. 2017).

Energetic demands for sperm motility can result in the production of ROS if high demands of ATP have to be produced through oxidative phosphorylation (Aitken and Curry 2011). However, the energy metabolism we found to be linked to sperm velocity represent an alternative pathway for ATP generation that minimizes ROS production. Lambda-crystallin is part of the pathway that converts D-glucuronate to D-xylulose-5-phosphate, an intermediate in the pentose phosphate pathway (PPP) (Wamelink et al. 2008, Asada et al. 2010), and was found in higher abundance in males with faster sperm (Table 5.S3). The PPP produces several glycolytic intermediates (Wamelink et al. 2008) and can also contribute to defence against reactive oxygen species (ROS) via production of NADPH, a major cofactor for antioxidant enzyme activity (Cosentino et al. 2011). My findings are very similar to a recent study in honeybees (*Apis mellifera*), where sperm utilise aerobic respiration following ejaculation when competing with other ejaculates to generate ATP, but avoid ROS generation during the longer-term storage phase of sperm in the spermatheca by switching to anaerobic glyceraldehyde-3-phosphate metabolism (Poland et al. 2011, Paynter et al. 2017). Gombar et al. (2017) found that "hooknose" Chinook salmon males had greater abundance of AK and

superoxide dismutase in their seminal fluid than "jack" males, while jacks had greater levels of lactate dehydrogenase. Therefore, jacks that face higher sperm competition risk may be better able to utilise lactate as an energy source, converting it to pyruvate that presumably then enters the oxidative phosphorylation pathway (Gombar et al. 2017). Combined with our results, this suggests that males with different reproductive strategies in salmonids may alter the primary energetic pathways supported by seminal fluid, balancing the need for high energy output with generation of harmful ROS.

Nine of the proteins with significant trends in abundance have calcium mediated functions. Interlinked Ca^{2+} mediated signalling pathways are involved in the regulation of fish sperm motility (Dzyuba et al. 2017, Zilli et al. 2017). SPARC and EF-hand calcium-binding domain-containing protein 1 (EFCB1) were found in higher abundance in ejaculates with faster sperm. SPARC is a multifunctional glycoprotein that modulates interactions between cells and the extra-cellular matrix via binding to structural molecules (Brekken and Sage 2000). SPARC is associated with axonemes of epithelial cilia and has been hypothesised to be a Ca^{2+} mediated regulator of ciliary movement (Huynh et al. 2000, Sodek et al. 2002, Huynh et al. 2004). SPARC also binds to sperm and regulates part of the sperm maturation process in monotremes (Nixon et al. 2016). In red junglefowl SPARC was also one of the proteins recently found in greater abundance in ejaculates with high sperm velocity (Borziak et al. 2016). Outside of the ability to bind with Ca^{2+} the function of EFCB1 is unknown, however our results suggest it will be of great interest to establish the possible role that both SPARC and EFCB1 play as mediators of salmon sperm function.

Proteases and their inhibitors are a major functional group of SFPs (Mueller et al. 2004, LaFlamme and Wolfner 2013), and 67 are found in the Chinook salmon seminal fluid proteome (Chapter Four). The protease inhibitor Latexin, a metallocarboxypeptidase inhibitor was found in higher abundance in ejaculates with faster sperm (Table 5.S3). Latexin exhibits regulatory function on stem cell numbers and signalling pathways involved in pain sensitivity (Jin et al. 2006, Liang et al. 2007, Liang and Van Zant 2008) possibly regulated by Ca^{2+}/Calmodulin signalling pathways (Liang et al. 2007), Latexin might therefore be involved in calcium regulated sperm functions but its function in seminal fluid requires further research. Tissue Inhibitor matrix metalloproteinase 2 (TIMP2) was found in lower abundance in ejaculates with faster sperm (Table 5.S3). TIMP2 inhibits Gelatinase A (MMP2), a matrix

metalloproteinase that regulates multiple biological processes via the degradation of many different targets in the extracellular matrix (Sternlicht and Werb 2001). TIMP2 not only binds to the active form of MMP2 inhibiting its proteolytic activity but is also required for the conversion of the latent proenzyme form into its active form, thus playing multiple roles in the regulation of MMP2 activity (Wang et al. 2000). The activity of MMP2 correlates with increased sperm motility in canine ejaculates (Saengsoi et al. 2011, Warinrak et al. 2015). The exact function of MMP2 in seminal fluid that influences sperm motility has not yet been established, however migration of glial cells involves the redistribution of MMP2 to actin motile structures suggesting that MMP2 influences cell motility via interaction with integrins and the actin cytoskeleton (Ogier et al. 2006, Lorenc et al. 2015).

Ten proteins with significant trends in abundance were immune related, however there are no clear patterns of protein abundance, as different immune related proteins were significantly associated with both social phenotypes and ejaculates containing higher and lower sperm numbers. SFPs with immune function in fish seminal fluid are primarily acute phase proteins of the innate immune system, protecting sperm and the male reproductive tract prior to and during the spawning season (Ciereszko 2008, Ciereszko et al. 2013, 2017). In the context of sperm competition, SFPs involved in self/non-self recognition are of interest as they may mediate targeted negative effects on sperm from rival males, however evidence from our prior work suggests that such targeted negative effects have not evolved in Chinook salmon (Bartlett et al. 2017; Chapters Two & Three). It is possible that variation in abundance of immune proteins reflects immune responses mounted by some individuals, although no males showed outward signs of poor health, they were captured from a wild population. The negative correlation between sperm concentration and abundance of the immune protein vitellogenin may also be linked to differential exposure of these wild males to environmental factors prior to spawning. Vitellogenin is expressed as an immune protein in male fish (Shi et al. 2006, Li et al. 2008, Tong et al. 2010) but is also expressed in males upon exposure to environmental oestrogens that have a negative effect on male reproductive traits including sperm production (Sumpter 1995, Folmar et al. 1996, Panter et al. 1998, Flammarion et al. 2000).

Recent research shows that significant changes to the sperm proteome occur upon initiation of motility in common carp (*Cyprinus carpio*) (Dietrich et al. 2016). Another recent study found

significant differences in the sperm proteome between high and low fertility groups in two sturgeon species (*Acipenser baerii* and *A. schrenckii*) (Li et al. 2017). Combined, these studies demonstrate that proteins within sperm that include energy metabolism, antioxidant and Ca^{2+} linked functions play important roles during fish sperm motility. Furthermore, results from the recent comparison of seminal fluid proteomes in Chinook salmon males with alternate life-histories link the seminal fluid proteome to sperm competition risk, discovered differences in proteins with energy metabolism, antioxidant and proteolytic functions (Gombar et al. 2017). Our results are the first to correlate sperm velocity measurements with the seminal fluid proteome in an externally fertilising fish and we report significant proteins from these same functional groups. Taken together, this suggests that future research should focus on proteins with these functions to uncover the underlying mechanism behind investment in high quality ejaculates observed in Chinook salmon (Chapters Two and Three). Moving forward, an experiment that examines changes in the sperm proteome following incubation in seminal fluid from males with different sperm competition risk would be particularly useful in determining seminal fluid mediated changes to sperm physiology.

In summary, we detect significant differences in the seminal fluid proteome of male Chinook salmon in association with ejaculate quality traits known to influence male reproductive success during sperm competition. It is now well known that sperm competition is a potent evolutionary force shaping ejaculate composition across a wide range of taxa. We suggest future research exploring proteins in the seminal fluid is warrant to further to our understanding as to how males allocate components of an ejaculate and make sperm more competitive. In particular, of great interest, is interplay between SFPs involved in balancing energy production and ROS defence, and between Ca^{2+} mediated protein phosphorylation and ROS that can disrupt these signalling pathways. While much remains to be discovered, our results provide insight on the pivotal role that seminal fluid proteins play in reproductive biology and strategic investment in ejaculate quality.

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5.3 SUPPLEMENTARY TABLES

Table 5.S1: Proteins detected in chinook salmon seminal fluid that have abundances significantly correlated with male social status with separate analysis of data collected at each experimental stage (Stage 1: n = 17, Dominant = 9, Subdominant = 8; Stage 2: n= 15, Dominant = 9, Subdominant = 6). Localization is given as secreted as detected by TargetP (S) or described as extracellular in UniprotKB or literature (EC). 95% CI refers to 95% Confidence Interval.

Accession	Gene	Name	Protein function as described in literature	Loc.	Slope	P value	95% CI
Proteins with higher abundance in subdominant males in 1st experimental stage							
Q64HX9	CathY	Cathepsin Y	**Protein degradation/proteolysis:** Cysteine proteinase with unknown function in seminal fluid.	EC	0.32	0.02	0.10 – 0.54
P06350		Histone H1	**Immune function:** Secreted histone H1 and peptide derivatives have been identified as antimicrobial proteins in several fish species [1–4].	EC	0.58	0.02	0.16 – 1.00
C1BFZ2	UBE2N	Ubiquitin-conjugating enzyme E2 N	**Regulatory:** Carries out K-63 linked protein ubiquitination that does not target substrate for degradation but is involved in several other pathways including activation of kinases in signalling and regulating endocytosis [5]. Flagellar protein ubiquitination may regulate cell motility [6].	EC	0.63	0.01	0.20 – 1.06
B5DGT2	MLE3	Myosin light chain 3, skeletal muscle isoform	**Cell structural protein:** Regulatory component of the myosin complex that functions as a molecular motor to move actin filaments [7].	_	0.65	0.003	0.30 – 1.00
B5XAP1	PACRG	Parkin coregulated gene protein homolog	**Spermatogenesis:** Its exact function is unknown but an experiment using transgenic mice demonstrates that loss of PACRG results in infertility [8].	_	0.66	0.04	0.09 – 1.23
Proteins with higher abundance in subdominant males in 2nd experimental stage							
P69069		Histone H2B	**Immune function:** Immune challenge increases expression of histone H2B in European sea bass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) fish [4]. Antimicrobial activity of histone H2B has also been documented in the bovine female reproductive tract [9].	EC	0.75	0.019	0.22 – 1.28

B5X4I3	SH3L3	SH3 domain-binding glutamic acid-rich-like protein 3	**ROS defence:** Glutaredoxin domain containing protein. Glutaredoxins are glutathione-dependent oxidoreductases that regulate redox homeostasis [10,11].	EC	0.86	0.013	0.35 – 1.37
B5X202	ZN576	Zinc finger protein 576	**Unknown function**	EC	0.52	0.013	0.19 – 0.85
B5DGY4	RS3	40S ribosomal protein S3	**Translation:** a component of the 40S small ribosomal subunit.	_	0.41	0.012	0.16 – 0.67
Proteins with higher abundance in dominant males in 1st experimental stage							
B8R4G1	SHBGα	Sex hormone-binding globulin alpha	**Regulatory:** Transports sex-hormones (e.g. testosterone) and regulates their access to target tissues, thus influencing reproductive development [12]. SHBG polymorphism is associated with sperm concentration and motility in humans [13–15].	S	-0.69	0.03	-1.24 – -0.14
B8R4G1	SHBGβ	Sex hormone-binding globulin beta	**Regulatory:** Transports sex-hormones (e.g. testosterone) and regulates their access to target tissues, thus influencing reproductive development [12]. SHBG polymorphism is associated with sperm concentration and motility in humans [13–15].	S	-0.83	0.03	-1.47 – -0.18
B5DGU8	LOC 106587886	Proteasome (Prosome, macropain) 26S subunit, non-ATPase, 13	**Protein degradation/proteolysis:** Part of the ubiquitin proteasome pathway (UPP) that selectively degrades substrate proteins covalently bound to ubiquitin. The UPP is vital for spermatogenesis and acts as a quality control mechanism by selectively degrading dysfunctional sperm [16–18].	EC	-0.69	0.03	-1.22 – -0.16

F8LFR3	SERPINF2B	Serpin peptidase inhibitor, clade F, member 2B	**Protease inhibitor:** Regulates proteolysis, specifically inhibiting serine-type endopeptidase activity. SERPINF2 negatively regulates plasmin activity and therefore prevents fibrinolysis [19]. Its regulation of plasmin may also contribute to activation of the complement pathway [20]. **Immune function:** Expression of SERPINF2B was upregulated in rainbow trout (*O. mykiss*) liver tissue following infection with *Aeromonas salmonicida* [21].	EC	-0.47	0.01	-0.78 – -0.16
W5S0H9	GSONMT 00026285001	GTP-binding nuclear protein Ran-A-1	**Transport:** GTPase involved in transport of proteins and RNAs in both import and the export from the nucleus [22].	_	-0.67	0.02	-1.13 – -0.21
C0PUT9	KCC2D	Calcium/calmodulin-dependent protein kinase type II delta chain	**Regulatory:** Mediates Ca^{2+} signalling pathways by phosphorylation of a range of substrates in a Ca^{2+}/calmodulin dependent fashion [23]. Protein phosphorylation mediated by Ca^{2+} is linked to sperm motility initiation in salmon [24].	EC	-0.34	0.01	-0.53 – -0.14
B5X1X1	ANX13	Annexin	**Regulatory:** Annexins are a family of Ca^{2+} regulated membrane and phospholipid-binding proteins. Involved in cellular responses to elevated Ca^{2+} levels including membrane trafficking and regulating ion flux across membranes. Several annexins have been found to occur extracellularly, although the function of extracellular ANX13 is unknown [25–28].	EC	-0.31	0.02	-0.52 – -0.10
C0HAB7	SFRS5	Splicing factor, arginine/serine-rich 5	**mRNA processing:** Arginine/serine-rich splicing factors are an evolutionarily conserved family of proteins involved in the post-transcriptional modification of mRNA [29].	_	-0.42	0.01	-0.69 – -0.15
C0HAD5	LAMP2	Lysosome-associated membrane glycoprotein 2	**Transport:** Transmembrane protein involved in the uptake of proteins to the lysosome for degradation [30,31]. **Cell matrix adhesion:** LAMP2 is also expressed on the cell surface, where it may mediate cell surface adhesion [32,33].	S	-0.42	0.03	-0.77 – -0.07

B5X4T0	FSTL1	Follistatin-related protein 1	**Regulatory:** Regulates the inflammatory response via inhibition of the Activin A activity. Follistatin and its regulation of Activin activity is also important for regulating the sperm maturation and transit in mammalian epididymis [34,35]	S	-0.43	0.02	-0.72 – -0.13
B5X2I6	LXN	Latexin	**Protease inhibitor:** A metallocarboxypeptidase inhibitor [36]. Exhibits regulatory function on stem cell numbers and signalling pathways involved in pain sensitivity [37–39]. The activity of Latexin is also possibly regulated by Ca^{2+}/Calmodulin signalling pathways [38].	S	-0.49	0.03	-0.88 – -0.10
B5X9Z8	ATIF1	ATPase inhibitor, mitochondria	**Energy metabolism:** Inhibits reversed activity of mitochondrial ATP synthase that consumes ATP to maintain membrane potential when mitochondrial respiration is compromised [40]	M	-0.57	0.03	-1.02 – -0.11
Proteins with higher abundance in dominant males in 2nd experimental stage							
Q9DFG0		Haptoglobin 2	**Protein degradation/proteolysis:** Protein with serine-type endopeptidase activity involved in acute phase response [41].	EC	-0.45	0.024	-0.77 – -0.13

Table 5.S2: Proteins detected in chinook salmon seminal fluid that have abundances significantly correlated with male social status using data from both experimental stages (n = 17, Dominant = 9, Subdominant = 8). Localization is given as secreted as detected by TargetP (S) or described as extracellular in UniprotKB or literature (EC). 95% CI refers to 95% Confidence Interval.

Accession	Gene	Name	Protein function as described in literature	Loc.	Slope	P value	95% CI
Proteins with higher abundance in subdominant males							
B5XG37	ENDD1	Endonuclease domain-containing 1 protein	**Immune function:** Identified as upregulated in Japanese flounder (*Paralichthys olivaceus*) brain, kidney, spleen and intestine following exposure to formalin killed bacteria (*Edwardsiella tarda*). Levels of DNase activity also increased in infected fish [42,43].	S	0.28	0.041	0.03 – 0.53
P06350		Histone H1	**Immune function:** Secreted histone H1 and peptide derivatives have been identified as antimicrobial proteins in several fish species [1–4].	EC	0.64	0.002	0.27 – 1.01
B5X4I3	SH3L3	SH3 domain-binding glutamic acid-rich-like protein 3	**ROS defence:** Glutaredoxin domain containing protein. Glutaredoxins are glutathione-dependent oxidoreductases that regulate redox homeostasis [10,11].	EC	0.55	0.029	0.09 – 1.01
B5DGT2	MLE3	Myosin light chain 3, skeletal muscle isoform	**Cell structural protein:** Regulatory component of the myosin complex that functions as a molecular motor to move actin filaments [7].	_	0.42	0.003	0.17 – 0.67
B5XBK1	VATE1	V-type proton ATPase subunit E 1-like	**Transmembrane transport:** V-type ATPase that function in acidifying cellular compartments regulating pH and coupled transport of small molecules [44,45].	_	0.19	0.015	0.06 – 0.32
Proteins with higher abundance in dominant males							
B5DGU8	LOC 106587886	Proteasome (Prosome, macropain) 26S subunit, non-ATPase, 13	**Protein degradation/proteolysis:** Part of the ubiquitin proteasome pathway (UPP) that selectively degrades substrate proteins covalently bound to ubiquitin. The UPP is vital for spermatogenesis and acts as a quality control mechanism by selectively degrading dysfunctional sperm [16–18].	EC	-0.48	0.005	-0.79 – -0.18

F8LFR3	SERPINF2B	Serpin peptidase inhibitor, clade F, member 2B	**Protease inhibitor:** Regulates proteolysis, specifically inhibiting serine-type endopeptidase activity. SERPINF2 negatively regulates plasmin activity and therefore prevents fibrinolysis [19]. Its regulation of plasmin may also contribute to activation of the complement pathway [20]. **Immune function:** Expression of SERPINF2B was upregulated in rainbow trout (*O. mykiss*) liver tissue following infection with *Aeromonas salmonicida* [21].	EC	-0.32	0.013	-0.55 – -0.08
B5X1H4	NHLC3	NHL repeat-containing protein 3	**Immune function:** NHLC3 in humans is involved in neutrophil degranulation, the regulated exocytosis of secretory granules from neutrophils as part of the innate immune response (Reactome ID: R-HAS-6806184).	S	-0.41	0.045	-0.78 – -0.03
B5XG91	PPT1	Palmitoyl-protein thioesterase 1	**Protein modification:** Enzyme that facilitates protein depalmitoylation, the removal of 16-carbon fatty acid chains from cysteine residues of substrate proteins. Protein palmitoylation/depalmitoylation regulates endocytosis of many proteins, the localisation and activity of small GTPases involved in signalling, and protein stability by preventing protein ubiquitination [46–48].	S	-0.25	0.014	-0.43 – -0.01
C0PUT9	KCC2D	Calcium/calmodulin-dependent protein kinase type II delta chain	**Regulatory:** Mediates Ca^{2+} signalling pathways by phosphorylation of a range of substrates in a Ca^{2+}/calmodulin dependent fashion [23]. Protein phosphorylation mediated by Ca^{2+} is linked to sperm motility initiation in salmon [24].	EC	-0.2	0.019	-0.35 – -0.04

B5X1X1	ANX13	Annexin	**Regulatory:** Annexins are a family of Ca^{2+} regulated membrane and phospholipid-binding proteins. Involved in cellular responses to elevated Ca^{2+} levels including membrane trafficking and regulating ion flux across membranes. Several annexins have been found to occur extracellularly, although the function of extracellular ANX13 is unknown [25–28].	EC	-0.26	0.003	-0.41 – -0.1
B8R4G1	SHBGα	Sex hormone-binding globulin alpha	**Regulatory:** Transports sex-hormones (e.g. testosterone) and regulates their access to target tissues, thus influencing reproductive development [12]. SHBG polymorphism is associated with sperm concentration and motility in humans [13–15].	S	-0.45	0.023	-0.82 – -0.09
C0HAB7	SFRS5	Splicing factor, arginine/serine-rich 5	**mRNA processing:** Arginine/serine-rich splicing factors are an evolutionarily conserved family of proteins involved in the post-transcriptional modification of mRNA [29].	_	-0.32	0.024	-0.57 – -0.06
C1BHC3	ATOX1	Copper transport protein ATOX1	**Transport:** Essential for cellular copper homeostasis, the ATOX1 metallochaperone delivers copper to ATPases in the copper secretory pathway [49,50]. **ROS defence:** There is also evidence that ATOX1 can protect cells from oxidative stress [51,52].	_	-0.35	0.046	-0.68 – -0.02
W5S0H9	GSONMT 00026285001	GTP-binding nuclear protein Ran-A-1	**Transport:** GTPase involved in transport of proteins and RNAs in both import and the export from the nucleus [22].	_	-0.38	0.029	-0.7 – -0.06
C0HAD5	LAMP2	Lysosome-associated membrane glycoprotein 2	**Transport:** Transmembrane protein involved in the uptake of proteins to the lysosome for degradation [30,31]. **Cell matrix adhesion:** LAMP2 is also expressed on the cell surface, where it may mediate cell surface adhesion [32,33].	S	-0.39	0.012	-0.69 – -0.11
B5XDG6	EPD	Ependymin	**Cell matrix adhesion:** Protein with calcium binding activity that may play a role in Ca^{2+} homeostasis, regulating cell surface adhesion and cell regeneration [53–55].	S	-0.43	0.006	-0.72 – -0.15

Table 5.S3: Proteins detected in chinook salmon seminal fluid that have abundances significantly correlated with sperm velocity using data from both experimental stages (n = 17). Localization is given as secreted as detected by TargetP (S), mitochondrial as detected by TargetP (M), described as extracellular in UniprotKB or literature (EC), or described as an exosome component (EX).

Accession	Gene	Name	Protein function as described in literature	Target P	Slope	P value	95% CI
Protein abundance positively correlated with sperm velocity							
B5DGU8	LOC 106587886	Proteasome (Prosome, macropain) 26S subunit, non-ATPase, 13	**Protein degradation/proteolysis:** Part of the ubiquitin proteasome pathway (UPP) that selectively degrades substrate proteins covalently bound to ubiquitin. The UPP is vital for spermatogenesis and acts as a quality control mechanism by selectively degrading dysfunctional sperm [16–18].	EC	0.013	0.015	0.003 – 0.02
B5X2I6	LXN	Latexin	**Protease inhibitor:** A metallocarboxypeptidase inhibitor [36]. Exhibits regulatory function on stem cell numbers and signalling pathways involved in pain sensitivity [37–39]. The activity of Latexin is also possibly regulated by Ca^{2+}/Calmodulin signalling pathways [38].	S	0.012	0.035	0.002 – 0.02
B5DGF9	SPRC	SPARC	**Signal transduction:** A multifunctional glycoprotein that modulates interaction between cells and the extra-cellular matrix via binding to structural molecules [56]. Observed in association with axonemes of epithelial cilia and via EF-hand domain is hypothesised as being a Ca^{2+} regulator of ciliary movement [57–59].	S	0.014	0.003	0.005 – 0.02
B5X9M8	EFCB1	EF-hand calcium-binding domain-containing protein 1	**Calcium ion binding:** Protein with EF-hand domain that has unknown biological function. EF-hand domain confers Ca^{2+} binding activity [60].	EC	0.009	0.02	0.002 – 0.02

B5XBY3	PRDX1	Peroxiredoxin-1	**ROS defence:** Oxidative stress, caused by reactive oxygen species (ROS) is known to impair sperm function causing damage to DNA, proteins and lipids. ROS are generated by energy metabolism pathways and the action of leukocytes in immune response. PRDXs protect sperm from oxidative damage by scavenging ROS and may also regulate H_2O_2 mediated signalling pathways [61–64].	EC	0.008	0.049	0.0004 – 0.02
B5X6Z9	CRYL1	Lambda-crystallin	**Energy metabolism:** Converts L-gulonate into 3-dehydro-L-gulonate in the second step of the glucuronate pathway, an alternate glucose metabolic pathway that converts D-glucuronate into D-xylulose-5-phosphate, an intermediate in the pentose phosphate pathway (Reactome ID: R-HSA-5661270) [65,66].	_	0.006	0.023	0.001 – 0.002
B9EMK7	TCEA1	Transcription elongation factor A protein 1	**Transcription:** Elongation factor that regulates transcription by regulating RNA polymerase II [67].	_	0.007	0.045	0.0005 – 0.01
B5XBK1	VATE1	V-type proton ATPase subunit E 1-like	**Transmembrane transport:** V-type ATPase that function in acidifying cellular compartments regulating pH and coupled transport of small molecules [44,45].	_	0.006	0.012	0.002 – 0.01
Protein abundance negatively correlated with sperm velocity							
B5X8G7	TIMP2	Metalloproteinase inhibitor 2	**Protease inhibitor:** Inhibitor of matrix metalloproteinase 2 (MMP2, Gelatinase A). High MMP2 activity in canine seminal fluid has been positively correlated with the number of motile sperm. How MMP2 influences sperm motility is currently unknown, however MMP2 has known functional association with integrins and the actin cytoskeleton, while also influencing cell behaviour via degradation of extracellular matrix proteins [68–70].	S	-0.006	0.04	-0.01 – -0.001

C0H7Q9	CRIP1	Cysteine-rich protein 1	**Immune function:** Member of the LIM protein family, generally thought to regulate cell differentiation. Evidence from studies of mammals suggest that CRIP1 has an immune function via the regulation of cytokine production, with expression of CRIP1 associated with immune cells and tissues and increasing following immune challenge [71,72].	S	-0.007	0.042	-0.01 – -0.0005
B5X1X1	ANX13	Annexin	**Regulatory:** Annexins are a family of Ca^{2+} regulated membrane and phospholipid-binding proteins. Involved in cellular responses to elevated Ca^{2+} levels including membrane trafficking and regulating ion flux across membranes. Several annexins have been found to occur extracellularly, although the function of extracellular ANX13 is unknown [25–28].	EC	-0.005	0.044	-0.01 – -0.0004
B5X499	SEPT2	Septin-2	**Regulatory:** Septins are a family of GTP binding proteins that are associated with cell membranes and the cytoskeleton. SEPT2 is involved in sperm tail formation and septin deficiency leads to development of non-functional flagella [73,74]. SEPT2 also regulates the uptake of extracellular Ca^{2+} [75,76].	_	-0.006	0.039	-0.01 – -0.001
Q6R4A2	CA2	Cytoplasmic carbonic anhydrase	**Regulatory:** A metalloenzyme that catalyses the reversible conversion of CO_2 into HCO_3-**,** regulating cellular pH. In mammalian sperm HCO_3- stimulates the cAMP-mediated pathway that alters flagellar beat frequency and therefore regulates sperm velocity [77–79].	_	-0.013	0.045	-0.03 – -0.001
Q0H913	gnao1	Guanine nucleotide binding protein G(O), alpha subunit 1 splice variant b	**Signal transduction:** Guanine nucleotide-binding proteins (G proteins) are involved as modulators or transducers in various signalling pathways [80,81].	_	-0.026	0.028	-0.05 – -0.004

B5X2Q5	MTAP	S-methyl-5'-thioadenosine phosphorylase	**Metabolism, amino acid biosynthesis:** Involved in the breakdown of S-methyl-5'-thioadenosine (MTA), a major by-product of polyamine biosynthesis. Responsible for the first step in the methionine salvage pathway (see UniprotKB entry).	EC	-0.011	0.002	-0.02 – -0.005
B5X205	AATC	Aspartate aminotransferase	**Metabolism, amino acid biosynthesis:** Biosynthesis of L-glutamate from L-aspartate or L-cysteine. Aspartate aminotransferase activity in seminal fluid has been negatively correlated with measures of fertility in *O. mykiss*, and authors suggest that the origin of enzyme activity is due to release from damaged spermatozoa [82,83].	EC	-0.009	0.012	-0.02 – -0.003
B5X1Q9	NB5R3	NADH-cytochrome b5 reductase 3	**Metabolism:** Serves as electron donor for the ubiquitous electron carrier cytochrome b5, thus participating in a variety of metabolic pathways (IPR001834).	EX	-0.01	0.043	-0.02 – -0.001
B5DH06	VDAC2	Voltage-dependent anion channel 2-2	**Transmembrane transport:** Forms a channel through the mitochondrial outer membrane that allows diffusion of small hydrophilic molecules, thus providing transport of anions, cations, ATP and other metabolites into and out of the mitochondria, and is also involved in mitochondrial-mediated cell death [84,85].	M	-0.0089	0.047	-0.02 – -0.001
B5X1V0	STOM	Erythrocyte band 7 integral membrane protein (Stomatin)	**Integral membrane component:** A membrane protein associated with membrane microdomains termed lipid rafts [86,87].	EX	-0.011	0.04	-0.02 – -0.001

Table 5.S4: Proteins detected in chinook salmon seminal fluid that have abundances significantly correlated with sperm concentration using data from both experimental stages (n = 17). Localization is given as secreted as detected by TargetP (S), mitochondrial as detected by TargetP (M) or described as extracellular in UniprotKB or literature (EC).

Accession	Gene	Name	Protein function as described in literature	Target P	Slope	P value	95% CI
Protein abundance positively correlated with sperm concentration							
F8LFR3	SERPINF2B	Serpin peptidase inhibitor, clade F, member 2B	**Protease inhibitor:** Regulates proteolysis, specifically inhibiting serine-type endopeptidase activity. SERPINF2 negatively regulates plasmin activity and therefore prevents fibrinolysis [19]. Its regulation of plasmin may also contribute to activation of the complement pathway [20]. **Immune function:** Expression of SERPINF2B was upregulated in rainbow trout (*O. mykiss*) liver tissue following infection with *Aeromonas salmonicida* [21].	EC	0.002	0.031	0.0002 – 0.003
C1BEH9	H2A	Histone H2A	**Immune function:** Secreted proteins derived from histone H2A have been identified as an antimicrobial proteins in several fish species [88–90].	EC	0.001	0.019	0.0004 – 0.003
B2DBF2		Troponin I	**Regulatory:** Troponin I is the inhibitory component troponin, a protein that regulates Ca^{2+} mediated muscle contraction [91].	_	0.002	0.018	0.0004 – 0.002
B8R4G1	SHBGα	Sex hormone-binding globulin alpha	**Regulatory:** Transports sex-hormones (e.g. testosterone) and regulates their access to target tissues, thus influencing reproductive development [12]. SHBG polymorphism is associated with sperm concentration and motility in humans [13–15].	S	0.002	0.032	0.0004 – 0.004

B5X3I8	CAHZ	Carbonic anhydrase	**Regulatory:** A metalloenzyme that catalyses the reversible conversion of CO_2 into HCO_3-**,** regulating cellular pH. In mammalian sperm HCO_3- stimulates the cAMP-mediated pathway that alters flagellar beat frequency and therefore regulates sperm velocity [77–79].	EC	0.002	0.004	0.001 – 0.003
C0H9G4	RAB31	Ras-related protein Rab-31	**Signal transduction:** Regulators of vesicular transport within cells, Rab proteins play key roles in regulating metabolism and signalling pathways [92].	_	0.002	0.021	0.0003 – 0.003
B5XCB2	GLNA	Glutamine synthetase	**Metabolism, amino acid biosynthesis:** Biosynthesis of L-glutamine from L-glutamate (see UniprotKB entry).	_	0.001	0.019	0.0003 – 0.002
B9ELP5	SERB	Phosphoserine phosphatase	**Metabolism, amino acid biosynthesis:** Biosynthesis of L-serine via hydrolysis of phospho-L-serine (see UniprotKB entry).	_	0.001	0.035	0.0002 – 0.002
B5XEM0	HEM2	Delta-aminolevulinic acid dehydratase	**Metabolism:** Enzyme that is part of the heme biosynthesis pathway [93].	_	0.001	0.017	0.0003 – 0.002
B5XEU8	FRI3	Ferritin	**Iron homeostasis:** Functions in the binding and storage of iron in a soluble, non-toxic, readily available form [94,95].	EC	0.001	0.015	0.0003 – 0.002
Protein abundance negatively correlated with sperm concentration							
B5X834	NATTE	Nattectin	**Immune function:** A C-type lectin originally discovered in the venomous fish *Thalassophryne nattereri*. C-type lectins are Ca^{2+} dependent carbohydrate binding proteins that are involved in pathogen recognition and phagocytosis [96–99].	EC	-0.002	0.035	-0.004 – -0.0003

X5IE94	VTG	Vitellogenin	A protein expressed in females as a precursor to egg yolk proteins in teleost fish [100]. Expressed in male fish following exposure to environmental estrogens, that have several adverse effects on male reproductive success, reducing sperm production, altering male sexual behaviours and phenotypes, and increasing male mortality [101–104]. **Immune function:** Expressed in males of several fish species following immune challenge, Vitellogenin functions as an opsonin by binding to pathogens and promoting phagocytosis [105–107].	EC	-0.002	0.031	-0.003 – -0.0002
P11941		Lysozyme C II	**Immune function:** Primary immune function is lysing bacteria cells. Has antibacterial activity against the Gram-positive bacterium *Planococcus citreus* in *O. mykiss* [108]**.**	S	-0.004	0.024	-0.01 – -0.001
Q4QZ18		Complement factor H1 protein	**Immune function:** The Complement system is an important component of the innate immune response, mediating phagocytosis and cytolysis of pathogens, inflammation and also enhancing humoral immune response. Factor H proteins regulate alternate pathway activation of the Complement system [109–111].	S	-0.002	0.007	-0.003 – -0.001
B5X3P8	PEDF	Pigment epithelium-derived factor	**Regulatory:** Protein belonging to the non-inhibitory serpin family group [112]. Its described functions include inhibiting angiogenesis, neurotrophic activity and protecting cells from environmental stresses including oxidative stress [113,114]. **Signal transduction:** Negative regulator of the Wnt signalling pathway [115,116].	S	-0.002	0.009	-0.003 – -0.001

B5X6Y1	AFP4	Type-4 ice-structuring protein	**Regulatory:** Anti-freeze protein that lowers the freezing point of blood or other biological fluids by inhibiting the formation of water ice crystals (see UniprotKB: KW-0047).	S	-0.003	0.009	-0.005 – -0.001
Q6R4A2	CA2	Cytoplasmic carbonic anhydrase	**Regulatory:** A metalloenzyme that catalyses the reversible conversion of CO_2 into HCO_3-**,** regulating cellular pH. In mammalian sperm HCO_3- stimulates the cAMP-mediated pathway that alters flagellar beat frequency and therefore regulates sperm velocity [77–79].	_	-0.004	0.004	-0.007 – -0.002
B5X2Q5	MTAP	S-methyl-5'-thioadenosine phosphorylase	**Metabolism, amino acid biosynthesis:** Involved in the breakdown of S-methyl-5'-thioadenosine (MTA), a major by-product of polyamine biosynthesis. Responsible for the first step in the methionine salvage pathway (see UniprotKB entry).	EC	-0.002	0.011	-0.003 – -0.001
B5X4I3	SH3L3	SH3 domain-binding glutamic acid-rich-like protein 3	**ROS defence:** Glutaredoxin domain containing protein. Glutaredoxins are glutathione-dependent oxidoreductases that regulate redox homeostasis [10,11].	EC	-0.003	0.035	-0.005 – -0.0004
O42161	ACTB	Beta-actin	**Cell structural protein:** Component of the cytoskeleton and ubiquitously expressed in all Eukaryotic cells (see UniprotKB entry).	_	-0.003	0.006	-0.005 – -0.001

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CHAPTER SIX – GENERAL DISCUSSION AND FUTURE DIRECTIONS

6.1 GENERAL DISCUSSION

The work presented in this thesis has collectively explored the reproductive strategies that have evolved in response to sperm competition risk in male Chinook salmon (*Oncorhynchus tshawytscha*), focusing on the role that seminal fluid plays as a mediator of differential investment in ejaculate quality. This discussion chapter summarises how my doctoral research has advanced our understanding of post-copulatory sexual selection, highlighting the role of seminal fluid and the proteins it contains. The chapter also discusses future directions for research, including the detailed investigation of seminal fluid protein function in salmonid reproductive biology.

6.1.1 SYNTHESIS AND IMPLICATIONS OF RESEARCH

Sperm competition theory predicts that males will trade-off between energy expended making high quality ejaculates and obtaining mating opportunities, and that males will invest differentially in ejaculates with respect to sperm competition risk (Parker 1990, 1998, Wedell et al. 2002, Birkhead et al. 2009, Parker and Pizzari 2010). In agreement with these predictions, males of many species can make rapid adjustments to ejaculate quality in response to social cues that signals changing sperm competition risk, such as the presence of a male competitor (Kilgallon and Simmons 2005, Smith and Ryan 2011, Burger et al. 2015) or change in social status (Rudolfsen et al. 2006, Pizzari et al. 2007, Cornwallis and Birkhead 2007, Fitzpatrick et al. 2008, Kustan et al. 2011). These adjustments to ejaculate quality can involve altering the performance of sperm, for example sperm velocity or viability, over time scales much faster than spermatogenesis (Kilgallon and Simmons 2005, Rudolfsen et al. 2006, Pizzari et al. 2007, Pizzari 2017). This suggests that males may alter the composition of seminal fluid to mediate changes in existing sperm (Simmons and Fitzpatrick 2012, Fitzpatrick and Lüpold 2014). However, the underlying mechanisms behind changes in ejaculate quality for most species are poorly understood, and research to date has yet to convincingly demonstrate that rapid alterations of sperm quality traits are mediated by seminal fluid.

My thesis combines research across a range of disciplines using Chinook salmon, an externally fertilising fish, in which males adopt alternative reproductive tactics that experience different levels of sperm competition risk (Berejikian et al. 2000, 2010, Esteve 2005). Chinook salmon display three different life history strategies; they can mature < 1 years or at 2 years of age, termed "precocious parr" and "jack" males respectively and adopt a tactic that tries to sneak fertilisations with females (Esteve 2005). Alternatively, males can mature when fully grown at 3+ years, termed "hooknose" and fight to establish social dominance and prime mating position next to a spawning female (Esteve 2005). Socially dominant males adopt a guarding tactic and attempt to monopolise access to spawning females, while subdominant hooknoses will adopt a sneaking tactic, whereby they must "sneak" into female nests for access to ova (Esteve 2005). Males with a sneaking tactic have a higher sperm competition risk, as their sperm will always be competing with sperm from a dominant male, and typically dominant males must also trade-off energy between fighting to secure territory and producing sperm and non-sperm components of an ejaculate. Due to the constraints of a fixed energy budget and different information about sperm competition risk available for each male; sperm competition theory predicts that dominant males will produce less competitive ejaculates than the other male phenotypes adopting the sneaking tactic (Parker 1990, Parker and Pizzari 2010).

In **Chapter Two**, I used a series of experiments on hooknose Chinook salmon to: 1) assess the rapid adjustment of ejaculate quality in response to change in male social status that signals changing sperm competition risk, 2) determine the role of seminal fluid in the underlying mechanism behind adjustment in ejaculate quality, and 3) establish whether these rapid adjustments to ejaculate quality ultimately influence a male's reproductive success under sperm competition conditions. Results from my experimental two-stage manipulation of male social status combined with measurement of ejaculate quality traits showed that, on average, subdominant males produced ejaculates with greater numbers of sperm and sperm with faster swimming speed. This trend was driven by a significant increase in sperm velocity observed in males that changed from dominant to subdominant social status within only 48-hours. The next experiment that separated and recombined sperm and seminal fluid from males of different social status, found that seminal fluid mediated changes to sperm velocity. Incubating the sperm of a dominant male in the seminal fluid of a subdominant male resulted

in an increase in sperm velocity, and likewise subdominant sperm incubated in the seminal fluid of a dominant male reduced their swimming speed. Finally, my *in-vitro* sperm competition trials that raced ejaculates from different males, counting the offspring sired by each male, determined that relative sperm velocity between competitors could predict the outcome of sperm competition, and that changes to sperm velocity caused by seminal fluid altered the number of offspring sired by each male and thus male reproductive success.

These results provide unequivocal evidence that seminal fluid mediates rapid changes to sperm velocity, that directly influence male fitness and resulted in a manuscript that was published in *eLife*. In his 'Insight' article that accompanied that paper, Pizzari (2017) concludes with the following insightful questions: 1) Can males exploit the seminal fluid of rival males for their own benefit? 2) Can seminal fluid discriminate between own and rival sperm? and 3) Which molecules in seminal fluid are involved?

Chapter Three addresses the first two questions raised by Pizzari (2017). A key result from the manipulation of ejaculates I performed in Chapter Two, was that the relative difference in sperm velocity between the males in each pair, is a better predictor of the effect that seminal fluid had on sperm velocity than male social status. In other words, males investing in higher quality ejaculates produced seminal fluid that increased the speed of sperm from males with lower quality ejaculates, regardless of male social status. This result suggests that seminal fluid does not discriminate between sperm from different hooknose males in Chinook salmon. Conversely, using a similar method of recombining sperm and seminal fluid, a study using the grass goby (*Zosterisessor ophiocephalus*) (Locatello et al. 2013), and a more recent study in Chinook salmon (Lewis and Pitcher 2017) that compared males adopting sneaker or guard tactics based on different life-histories. These studies compared group averages across seminal fluid treatments (i.e., sperm in own vs in rival seminal fluid) and found that seminal fluid from sneaker males decreased the velocity of sperm from guards, while having no effect (Locatello et al. 2013) or increasing velocity of sperm from males of the same tactic (Lewis and Pitcher 2017).

To further investigate whether seminal fluid from males with alternate life-histories can discriminate between sperm from different males, in **Chapter Three** I recombined ejaculates between hooknose and precocious parr males and compared the analytical approach used in Chapter Two with that of the studies discussed above (Locatello et al. 2013, Lewis and Pitcher

2017) also reanalysing the data from Lewis and Pitcher (2017). By Comparing averages across seminal fluid treatment groups (i.e., sperm incubated in own or rival seminal fluid) I revealed a significant negative effect of hooknose seminal fluid on precocious parr sperm. This result makes little evolutionary sense, as it would suggest that males with lower sperm competition risk have invested in ejaculates to combat sperm from males with higher sperm competition risk. However, as in Chapter Two, utilising the alternate approach I found a significant linear relationship between relative difference in sperm velocity between males and the change in velocity cause by seminal fluid, a trend also found when reanalysing the data from Lewis and Pitcher (2017). Combined with Chapter Two, this work assesses data from males of all three life-history strategies in Chinook salmon and strongly suggests that targeted negative effects on rival sperm have not evolved in this species. This likely reflects the exceptional set of conditions that characterise sperm competition in salmonids, where sperm must find and fertilise an egg within an incredibly short time frame in an external environment (Hoysak and Liley 2001, Yeates et al. 2007). These conditions provide strong selection for fast swimming sperm while also likely constraining the evolution of interaction between ejaculates from different males, preventing both discriminatory effects against rivals and the exploitation of rival ejaculates.

Chapter Four and **Chapter Five** work towards the third question raised by Pizzari (2017; *Which molecules in seminal fluid are involved?)* and focus on the Chinook salmon seminal fluid proteome. Seminal fluid proteins (SFPs) have been associated with sperm function in a number of species (Poiani 2006, Simmons and Fitzpatrick 2012, Perry et al. 2013, Fitzpatrick and Lüpold 2014) and are known to play critical roles that influence the outcome of sperm competition, particularly in insects (den Boer et al. 2010, 2015, Avila et al. 2011, Sirot et al. 2015). Our understanding of SFPs in externally fertilising fish however is comparatively lacking, and the complex role that SFPs play in reproduction, sperm physiology and function for fish is only now beginning to be unravelled. The overall aim of the proteomic work I conducted in thesis research, was to identify candidate proteins involved in sperm and seminal fluid interaction that may mediate seminal fluid effects on sperm velocity in Chinook salmon. However, before such quantitative proteomic work can be meaningfully interpreted, the critical first step is to generate a list of identified proteins and characterise that list in detail.

Research to describe the seminal fluid proteome in fish has been conducted for both common carp (*Cyprinus carpio*) (Dietrich et al. 2014) and rainbow trout (*Oncorhynchus mykiss*) (Nynca et al. 2014), identifying 137 and 152 SFPs respectively. Most recently, Gombar et al. (2017) quantified differences in SFP abundance between hooknose and jack Chinook salmon males identifying 345 SFPs present in both tactics and found that 21 proteins differed in abundance between the two male phenotypes, including proteins involved in energy metabolism, redox regulation and immune function that may influence sperm function. In **Chapter Four**, I use a combination of prefractionation techniques, followed by LC-MS/MS analysis and exclusion list searching (Eubel et al. 2008) to improve the number of proteins detected in Chinook salmon seminal fluid, using samples collected during the social status manipulation from Chapter Two. I then assigned biological function of proteins using database and literature searching and conducted the first inter- and intra- species comparative analysis for seminal fluid proteomes of teleost fish.

I present a high confidence list of 549 proteins identified in Chinook salmon seminal fluid and report 378 proteins not previously detected in Chinook salmon seminal fluid. Nearly half (47%) of the identified proteins are associated with metabolic processes, with 25% involved in the metabolism of proteins and amino acids, 67 of which are proteases/protease inhibitors representing 12% of the total proteome. Consistent GO profiling with previous research combined with the highest Mascot scoring proteins representing abundant SFPs previously detected in teleost's (Nynca et al. 2014, Dietrich et al. 2014, Gombar et al. 2017), further supports that seminal fluid proteomes and the proposed major functional roles of seminal fluid are highly conserved among teleost species; to protect spermatozoa within the testes and during fertilisation, and to regulate key physiological processes such as energy metabolism and motility (Ciereszko 2008, Ciereszko et al. 2013).

Chapter Four also outlines several functional groups of proteins that are possible mediators of sperm function detected in the Chinook salmon seminal fluid proteome. These include proteins involved in the calcium mediated signalling pathways that trigger protein phosphorylation cascades to regulate cell motility (Dzyuba et al. 2017, Zilli et al. 2017). Proteases and their inhibitors that play key roles in the regulation of several reproductive processes including semen coagulation, eliciting post-mating responses in females, immune response within the reproductive tract, sperm maturation and activation of sperm motility

(Smith and Stanfield 2011, LaFlamme et al. 2012, Zhao et al. 2012, LaFlamme and Wolfner 2013, Dietrich et al. 2017). Proteins that alter energy metabolism and the availability of ATP that powers flagellar movement (Christen et al. 1987, Dzyuba et al. 2016, 2017) and proteins with antioxidant activity that can protect sperm for the harmful effects of reactive oxygen species (ROS) (Aitken and Curry 2011, Gao et al. 2017).

In **Chapter Five**, I quantified the mass spectra for 348 proteins using a spectral counting technique to generate relative abundance values. For each of these proteins I used statistical models to determine if male social status, sperm velocity or sperm concentration could predict protein abundance. A total of 57 proteins were associated with at least one of the three predictor variables, of those, 38 proteins were classified as extracellular. I found that 26 proteins differed significantly in abundance between dominant and subdominant males, 8 proteins were positively correlated while 10 proteins were negatively correlated with sperm velocity, and 10 proteins were positively correlated while 10 were negatively correlated with sperm concentration. Overall, the gene ontology of these proteins varied across 17 functional groups, with the largest groups including 10 with immune function and 9 with regulatory functions, as well as 4 protease inhibitors. SFPs with functions linked to sperm motility, energy metabolism, and anti-oxidant activity were detected and are discussed as candidates for future research on the molecular interaction between sperm and seminal fluid in externally fertilising fish and other vertebrate species.

6.2 FUTURE DIRECTIONS

Chapter Five provides a candidate list of seminal fluid proteins that may be linked to sperm function in Chinook salmon and perhaps in other vertebrates, therefore providing a platform from which future research can be based. Furthermore, during the course of this thesis research, questions have arisen that remain unanswered and interests piqued outside of the scope of this project. Rather than provide an exhaustive list on what should be done next, the following four sections highlight some areas of interest for future research that, given the opportunity, I would explore further.

6.2.1 EXPLORING ADDITIONAL SOCIAL CUES

This first section starts with a valuable lesson learned; in research, especially when dealing with populations of wild animals, things don't always go as planned. When I started this

project in 2014, my co-supervisors encouraged me to think of a small experiment that could be conducted in parallel with the main social status manipulation experiment reported in Chapter Two. Some of the first papers that I studied in detail were those that experimentally manipulated the social status of male fowl (*Gallus gallus*), assessing ejaculate investment strategies and ejaculate plasticity in these birds (Cornwallis and Birkhead 2006, 2007, Pizzari et al. 2007). Something that stood out in these experiments was that males made investments not only based upon sperm competition risk but also perceived female quality (Cornwallis and Birkhead 2006, 2007). Differential investment in ejaculates based on perceived female quality in salmonids at the time had never been examined (but see Makiguchi et al. 2016). Thus, I devised a side project to test the hypothesis that male Chinook salmon increased their investment in ejaculates when paired with a female of perceived high quality.

In a review of salmonid spawning behaviour, Esteve (2005) describes some precopulatory mate choice by male hooknose salmon that will preferentially spawn with females of larger size. Research has shown that larger female salmon are more fecund, producing more and larger ova (de Eyto et al. 2015, Makiguchi et al. 2016, Thorn and Morbey 2018). It is possible then that body size represents an honest signal of female quality in salmonids. Using body size as a proxy for female quality, an experimental design was developed as follows:

The ejaculate reserves of a hooknose male were depleted prior to the experiment so that ejaculates were produced during the experiment. That male was then placed in isolation within an enclosure for two days, after which milt was collected as described in Chapter Two. A “small” female was introduced to the enclosure and following two days of interaction milt was again collected. The “small” female was then removed and replaced with a “large” female and following two days of interaction milt was again collected. An additional cycle of the “small” then “large” female interaction was conducted over a further four days, with milt collected after each two-day period of interaction with a different sized female, with each trial lasting a total of ten days. At each point where milt was collected, measurement of sperm velocity and sperm concentration was conducted as described in Chapter Two. There was a difference in fork length of > 10 cm between the small and large female.

In 2014, many salmon returned to spawn and we had the resources to conduct three ten-day trials, the results for which are presented in Figure (6.1). Unfortunately, the number of fish returning in 2015 and 2016 was low and I was forced to abandon this project, unable to

conduct any further trials and still complete the main experiments reported in this thesis. While drawing conclusions from a sample size of three would be premature, the results that were obtained are promising and suggest that future experiments of this nature would be worthwhile. Indeed, Makiguchi et al. (2016) estimated the number of sperm released by males using data-loggers to record vibration patterns in male chum salmon (*O. keta*) and show that the estimated number of sperm released by males is correlated with female size. This experiment was designed to be straightforward in that it only assessed male preference, using the initial period of isolation to remove influence of prior interactions with other males. In future work however, it would be fascinating not only to establish whether male Chinook salmon adjust their ejaculate quality with respect to perceived female quality but also whether such adjustments differ among males depending on their social status or life-history. The experimental design whereby males cycle through interaction with females of different size would also help to answer questions around how males allocate ejaculates with respect to future mating opportunities.

6.2.1.1 ETHICS

All animals were collected and maintained according to the approved standards of the Animal Ethics Committee for the University of Otago, New Zealand.

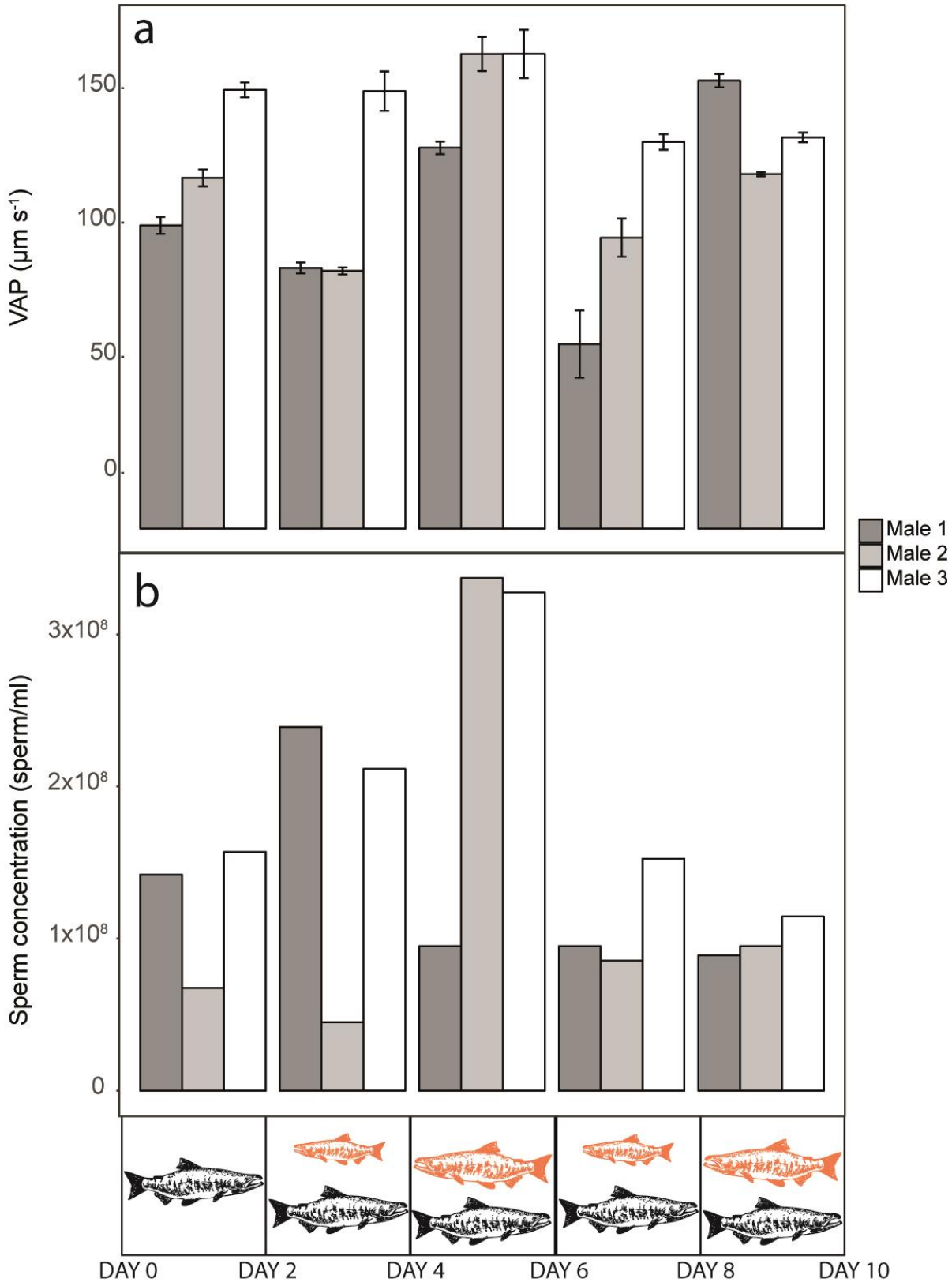

Figure 6.1: Male preference experiment in Chinook salmon, in which a hooknose male is placed in an initial two-day isolation period and then exposed to females of different size, switching between a small and then large female in two-day blocks over a total period of ten days. a) shows sperm velocity (VAP µm s^{-1}) and b) sperm concentration (sperm/ml) for three males.

6.2.2 SPERM STAINING AND NATURAL EJACULATES IN SALMONIDS

The results presented in Chapter Three support the idea that Chinook salmon males investing in high quality ejaculates produce seminal fluid that provides a similar benefit to sperm from any male. As discussed in Chapter Three and in the discussion above, this result differs from that published in two previous studies that report "tactic specific" effects of seminal fluid from early maturing sneaker males on the sperm of later maturing guard males (Locatello et al. 2013, Lewis and Pitcher 2017; Chapter Three). While the results of the experiment in Chapter Three, including the reanalysis of data from Lewis and Pitcher (2017), demonstrate that targeted negative effects of seminal fluid on rival sperm are highly unlikely in salmonids, the use of differential sperm staining and obtaining velocity measurements from different male's sperm activated simultaneously in the same seminal fluid would fully resolve this argument.

Sperm staining methods to visualisation and identify sperm from different males such as those methods used in *Drosophila,* resolved whether sperm incapacitation effects (Snook and Hosken 2004, Manier et al. 2010, Okada and Hosken 2010), and in squid (*Loligo bleekeri*) demonstrated differential chemotactic behaviours of sperm from males with alternative reproductive tactics (Hirohashi et al. 2013). The use of sperm staining has been applied to determine relationships between sperm morphometry and motility parameters in rainbow trout (*Oncorhynchus mykiss*) (Tuset et al. 2008a, 2008b); however, these methods apply stain to a subset of sperm from an ejaculate that are killed in the process and sperm motility parameters were then obtained from another sample of sperm from the same ejaculate. Development of a sperm staining method that can be applied to live salmonid sperm without initiating motility would likely be challenging. However, this would enable a range of innovative experiments, that could resolve fully whether targeted negative effects on sperm velocity by seminal fluid occur in salmonids and additionally further investigate the unknown mechanisms behind cryptic female choice exerted by ovarian fluid (Rosengrave et al. 2008, 2016, Egeland et al. 2016).

Another methodology that would provide valuable insight into ejaculate allocation by male salmonids is the collection of natural ejaculates. Natural ejaculates are likely to differ considerably from those collected manually by researchers. For example, male fowl can allocate ejaculates with more or less sperm and sperm with different velocity at mating after

assessing females (Pizzari et al. 2003, Cornwallis and Birkhead 2006, 2007). In addition, proteins in seminal fluid that are ejaculated naturally likely differ from those in manually collected ejaculates, as SFPs transferred to females differ from the total subset of proteins identified in male reproductive tracts (Findlay et al. 2008, Dean et al. 2011), and males can alter their allocation of SFPs at mating (Wigby et al. 2009, Sirot et al. 2011).

Collection of natural ejaculates in salmonids, and external fertilisers in general, poses a challenge. Ejaculates cannot be retrieved from females as in internally fertilising species and cannot be retrieved once released into fast flowing water. Furthermore, exposure to water will activate sperm motility and prevent further analysis of sperm velocity. Despite these challenges, Fitzpatrick and Liley (2008) developed a method where a piece of rubber tube was surgically attached over the gonopore of male rainbow trout, to which a condom could be fastened. They found that ejaculate volume (i.e. the number of sperm) was not influenced by the presence of a rival male (Fitzpatrick and Liley 2008). More recent research using an adaption of this method found that chum salmon (*O. keta*) males allocated more sperm to larger females (Makiguchi et al. 2016). Using a similar method to establish if male salmonids allocate ejaculates with different sperm velocity, or alter seminal fluid components, in response to sperm competition risk and female quality would demonstrate that complex ejaculate allocation strategies have evolved in a range of taxa that includes externally fertilising fish.

6.2.3 FURTHER PROTEOMIC STUDIES

While the looking at both the sperm and seminal fluid proteome was outside the scope of this thesis, a promising direction for future research would be to assess changes in sperm proteomes under different conditions. For example, in the honeybee (*Apis mellifera*) comparing the proteome of ejaculated sperm and sperm from the queens spermatheca revealed differences in proteins associated with energy metabolism (Poland et al. 2011). More recently, comparison of proteomes from common carp (*Cyprinus carpio*) sperm that were either immobilised or actively motile identified a suite of proteins that change in abundance in sperm associated with motility (Dietrich et al. 2016). This kind of approach where sperm are incubated in seminal fluid from different males and the proteome is then analysed would yield valuable information about the way sperm react to seminal fluid from different males. This approach would provide much needed information about sperm

physiology in the context of sexual selection in externally fertilising fish. A similar approach where sperm are activated in both water and ovarian fluid would assess the influence of ovarian fluid on the sperm proteome and possibly uncover unknown mechanisms of cryptic female choice.

6.2.4 TAKING A MORE TARGETED APPROACH

The results discussed in Chapter Five outline several proteins of interest for future research that were correlated with sperm velocity. In particular, the protease inhibitors Latexin and Tissue Inhibitor of Matrix Metalloproteinase 2 (TIMP2), the calcium binding proteins SPARC and EF-hand calcium-binding domain-containing protein 1 (EFCB1), and the antioxidant protein Peroxiredoxin-1 were identified. Before any functional assessment of these proteins is made however, antibody-based methods of protein quantitation, such as Western blotting or enzyme linked immunosorbent assays (ELISA) (see Sirot et al. 2009), should be used to validate the results from the proteomic work. Moving forward, research to assess the function of these proteins and their influence on salmonid sperm velocity, possibly by inhibiting their function *in-vitro* and measuring effects on sperm motility and physiology, is required to establish the role of these proteins in sperm competition. For example, antibodies developed for SPARC could be used to block its function (Purcell et al. 1993, Sweetwyne et al. 2004) and chemical inhibitors could be used to block the activity of peroxiredoxin (Ryu et al. 2017) or inhibit activity of matrix metalloproteinase 2 to mimic the effects of TIMP2 (Ogier et al. 2006). The evolution of reproductive traits in Chinook salmon has clearly been influenced by sperm competition and external fertilisation provides an opportunity to experimentally manipulate sperm and SFPs in an environment that is biologically relevant. Therefore, Chinook salmon provide an excellent model system for future research on the role of post-copulatory sexual selection in shaping SFP function.

6.3 CONCLUDING REMARKS

My doctoral research represents a significant advance in our understanding of post-copulatory sexual selection and the evolution of adaptations to sperm competition risk. Using a combination of behavioural manipulation, ejaculate manipulation, in vitro sperm competition experiments and proteomic analyses on chinook salmon males with alternative reproductive tactics, I provide unequivocal evidence that sperm competition risk drives patterns of investment in ejaculate quality. Collectively, this body of work demonstrates the

importance of seminal fluid in the reproductive biology of an externally fertilising fish and contributes to a growing body of literature that highlights the way in which sexual selection drives the adaptive evolution of the entire ejaculate.

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APPENDIX A:

CHAPTER TWO: PUBLISHED MANUSCRIPT

The following pages contain the published PDF version of the manuscript arising from Chapter Two:

Bartlett, M.J., Steeves, T.E., Gemmell, N.J., and Rosengrave, P.C. 2017. Sperm competition risk drives rapid ejaculate adjustments mediated by seminal fluid. *eLife* **6**: e28811. doi:10.7554/eLife.28811.

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RESEARCH ARTICLE

Sperm competition risk drives rapid ejaculate adjustments mediated by seminal fluid

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Abstract In many species, males can make rapid adjustments to ejaculate performance in response to sperm competition risk; however, the mechanisms behind these changes are not understood. Here, we manipulate male social status in an externally fertilising fish, chinook salmon (*Oncorhynchus tshawytscha*), and find that in less than 48 hr, males can upregulate sperm velocity when faced with an increased risk of sperm competition. Using a series of *in vitro* sperm manipulation and competition experiments, we show that rapid changes in sperm velocity are mediated by seminal fluid and the effect of seminal fluid on sperm velocity directly impacts paternity share and therefore reproductive success. These combined findings, completely consistent with sperm competition theory, provide unequivocal evidence that sperm competition risk drives plastic adjustment of ejaculate quality, that seminal fluid harbours the mechanism for the rapid adjustment of sperm velocity and that fitness benefits accrue to males from such adjustment.
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Introduction

Sperm competition (***Parker, 1970***) occurs commonly across many invertebrate and vertebrate taxa and is a potent evolutionary force influencing male reproductive biology (***Birkhead and Møller, 1998***; ***Birkhead and Pizzari, 2002***; ***Simmons and Fitzpatrick, 2012***). Sperm competition theory predicts that males will trade-off between energy expended making high-quality ejaculates with obtaining mating opportunities and that males will invest differentially in ejaculates with respect to sperm competition risk (***Parker, 1990***; ***Parker et al., 1997***; ***Parker, 1998***; ***Wedell et al., 2002***; ***Birkhead et al., 2009***; ***Parker and Pizzari, 2010***). In agreement with these predictions, males of many species can make rapid adjustments to ejaculate quality within days (***Rudolfsen et al., 2006***; ***Pizzari et al., 2007***; ***Thomas and Simmons, 2007***; ***Gasparini et al., 2009***; ***Smith and Ryan, 2011***), hours (***Cornwallis and Birkhead, 2007a***) and even minutes (***Kilgallon and Simmons, 2005***; ***Joseph et al., 2015***) of exposure to a new social cue that signals changing sperm competition risk, such as the presence of a female, or a male competitor. For example, in fowl (*Gallus gallus*), males of dominant social status strategically allocate sperm, ejaculating more and faster swimming sperm in initial copulations and to females of higher quality (***Pizzari et al., 2003***; ***Cornwallis and Birkhead, 2006***; ***Cornwallis and Birkhead, 2007a***; ***Cornwallis and Birkhead, 2007b***), and alter their allocation strategy accordingly when changing social status (***Cornwallis and Birkhead, 2007a***). While males of several vertebrate species ranging from fish (***Rudolfsen et al., 2006***; ***Gasparini et al., 2009***; ***Smith and Ryan, 2011***) to humans (***Kilgallon and Simmons, 2005***; ***Joseph et al., 2015***) can strategically alter the quality of their ejaculate in response to social cues, the mechanism behind such rapid adjustments is as yet unknown.

A promising candidate mechanism for rapid adjustment of sperm velocity may be found in the non-sperm component (seminal fluid and its constituents) of the ejaculate, particularly if such

eLife digest Males of many animal species fight to establish social dominance and control access to females so that they have more opportunities to reproduce than their competitors. Males with lower social status will struggle to directly compete for mates, thus they attempt to mate with females by stealth. This often leads to more than one male mating with the same female so that the sperm from each male end up competing to fertilise that female's eggs, a phenomenon known as sperm competition.

Males suspend their sperm in a fluid to make a mixture known as semen. It has been shown that, compared to high status males, low status males will produce higher quality semen that contains greater numbers of faster swimming sperm, giving them an advantage in sperm competition. Growing evidence from several species indicates that males can quickly adjust how fast their sperm swim in response to social cues that signal changing risks of sperm competition. However, how these rapid adjustments occur remains largely unknown, and whether they alter a male's reproductive success against a competitor has seldom been examined.

Chinook salmon usually live in the North Pacific Ocean but they swim up rivers in North America and Asia to reproduce. They have also been introduced to several other countries including New Zealand where they are farmed commercially. The fish are highly prized by sport fishermen and are also of cultural significance to certain groups of indigenous people in North America. Barlett et al. studied the semen of chinook salmon, undertaking a series of experiments in which males switched between high and low social status. The experiments show that, as predicted, the sperm of males that changed from high to low social status started to swim faster. These changes in speed were caused by the fluid in the semen and altered the number of eggs that the male's sperm fertilised when competing against sperm from another male.

In their natural range some populations of chinook salmon are declining due to overfishing combined with habitat loss and alteration. The findings of Barlett et al. contribute to a better understanding of how this fish species reproduces, which may lead to the introduction of measures that help natural populations to recover or help to improve commercial farming. Improved knowledge of how the fluid in semen affects sperm activity may also have important consequences for our wider understanding of male fertility in humans and other animals.

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adjustments occur more rapidly than spermatogenesis (***Cameron et al., 2007***; ***Perry et al., 2013***; ***Fitzpatrick and Lüpold, 2014***). Seminal fluid is a complex medium containing a great diversity of molecules (***Poiani, 2006***; ***Juyena and Stelletta, 2012***) and is known to influence sperm velocity and motility in vertebrates (***Lahnsteiner et al., 1998***; ***Lahnsteiner et al., 1996***; ***Poiani, 2006***; ***Locatello et al., 2013***; ***González-Cadavid et al., 2014***). For example, research using an externally fertilising fish, the grass goby (*Zosterisessor ophiocephalus*), compared males for which sperm competition strategy is determined by age/size and found large males that adopt a guarding strategy have a greater concentration of the seminal fluid glycoprotein mucin (***Scaggiante et al., 1999***). Furthermore, by separating and recombining seminal fluid and sperm from different males, research using the same species found seminal fluid had a tactic-specific effect on sperm velocity, with seminal fluid from sneak males decreasing the velocity of rival guard male sperm and seminal fluid from guard males increasing the velocity of sneak male sperm (***Locatello et al., 2013***).

However, only one study to date has investigated the role that seminal fluid plays as a mediator of short-term plastic sperm performance in a vertebrate species using fowl and the results were inconsistent with theoretical expectation: ***Cornwallis and O'Connor, 2009*** found that while ejaculates produced by male fowl that were allocated to females of higher quality contained faster sperm, seminal fluid from those ejaculates reduced the velocity of sperm from the same male allocated to females of lower quality. To be consistent with the prediction that seminal fluid mediates changes in sperm velocity, seminal fluid from ejaculates allocated to higher quality females should increase, not decrease the speed of sperm isolated from ejaculates allocated to lower quality females. Thus, although there is evidence that seminal fluid can influence sperm velocity, evidence that seminal fluid

mediates the rapid plastic adjustment of an ejaculate's motile performance consistent with theoretical expectation is lacking.

We use an ideal model species, chinook salmon (*Oncorhynchus tshawytscha*), to examine patterns of ejaculate plasticity in response to changes in male social status and the reproductive consequences of these changes. In salmonids, fertilisation occurs externally and sperm competition occurs in the majority of spawnings (**Berejikian et al., 2010**; **Sørum et al., 2011**). Male chinook salmon adopt Alternative Reproductive Tactics (ARTs) situationally, as 'hooknose' males fight to establish social dominance (**Esteve, 2005**). Only dominant males guard spawning females thus obtaining priority in mating position, while subdominant males that lose contests attempt to sneak fertilisations by invading spawning pairs and releasing their sperm (**Esteve, 2005**). The social status of male salmon is subject to change over the course of a spawning season; for example, in coho salmon (*O. kisutch*), 22% of observed contests between hooknose males resulted in displacement of the previous dominant male (**Healey and Prince, 1998**). Therefore, in this mating system, females mate with multiple males in a dynamic social environment that results in intense levels of fluctuating sperm competition risk.

Previous research has shown that when males engage in sperm competition, sperm swimming speed is the primary predictor of fertilisation success in chinook salmon (**Evans et al., 2013**; **Rosengrave et al., 2016**) and other salmonids (**Gage et al., 2004**; **Liljedal et al., 2008**; **Egeland et al., 2015**). Sperm competition theory therefore predicts subdominant males, which have greater sperm competition risk, will invest in ejaculates with faster swimming sperm than dominant males and males changing social status should adjust their investment accordingly (**Parker, 1990**; **Parker et al., 1997**; **Parker, 1998**; **Wedell et al., 2002**; **Birkhead et al., 2009**; **Parker and Pizzari, 2010**). Indeed, several studies that experimentally manipulated social status using Arctic charr (*Salvelinus alpinus)* have found that subdominant males produce ejaculates with more sperm and faster swimming sperm than dominant males (**Liljedal and Folstad, 2003**; **Rudolfsen et al., 2006**; **Vaz Serrano et al., 2006**; **Haugland et al., 2009**). Furthermore, **Rudolfsen et al. (2006)** demonstrated that following a social challenge, both sperm concentration and velocity decreased over a 4-day period compared with pre-trial levels in dominant males, and observed an increase in sperm concentration but no change in sperm velocity for subdominant males. However, **Rudolfsen et al. (2006)** did not evaluate male social status prior to the social challenge, so it is unknown if these males actually changed or simply retained the same status through the course of the experiment. Recent research shows that ejaculates from subdominant Arctic charr sire the same number of eggs when in competition with ejaculates from dominant males if their sperm were released after the average delay observed under natural conditions (**Egeland et al., 2015**). These results suggest that salmonid males in disfavoured mating positions can compensate by producing more competitive ejaculates than dominant males, but whether males changing social status adjust their sperm velocity, and if such adjustments to ejaculates are mediated by sperm or non-sperm components of the ejaculate, is yet to be determined.

Here, we use a comprehensive experimental approach to determine if changes in sperm velocity observed in response to an individual's social position are the result of alterations to the gametes or to seminal fluid and if such responses actually alter a male's reproductive success against a sperm competitor. Specifically, we examine whether ejaculate quality is phenotypically plastic in response to changes in sperm competition risk over 48 hr periods, using a two-stage challenge to manipulate social status (**Cornwallis and Birkhead, 2007a**; **Pizzari et al., 2007**) and collected ejaculates at each stage of the experiment. In the second stage, males either retained or were forced to change their social status, creating four social phenotypes with varying sperm competition risk (**Figure 1**). We found that subdominant males, which have greater sperm competition risk, invest more in both sperm concentration and sperm velocity compared to socially dominant males. Additionally, we find males that change from dominant to subdominant social status, thus elevated their sperm competition risk, increased their sperm velocity as predicted by sperm competition theory (**Parker, 1990**; **Parker et al., 1997**; **Parker, 1998**; **Wedell et al., 2002**; **Birkhead et al., 2009**; **Parker and Pizzari, 2010**). We also separated sperm from seminal fluid and created reciprocal combinations both within and between rival males, finding that males can make rapid adjustments to sperm velocity by producing seminal fluid that enhances sperm function. We then used *in vitro* fertilisation trials and found the seminal fluid effects on sperm swimming speed influences male reproductive success under sperm competition. Our combined experimental results provide compelling evidence that seminal fluid is the mediator of rapid strategic adjustment of sperm velocity, thus bringing us a critical step

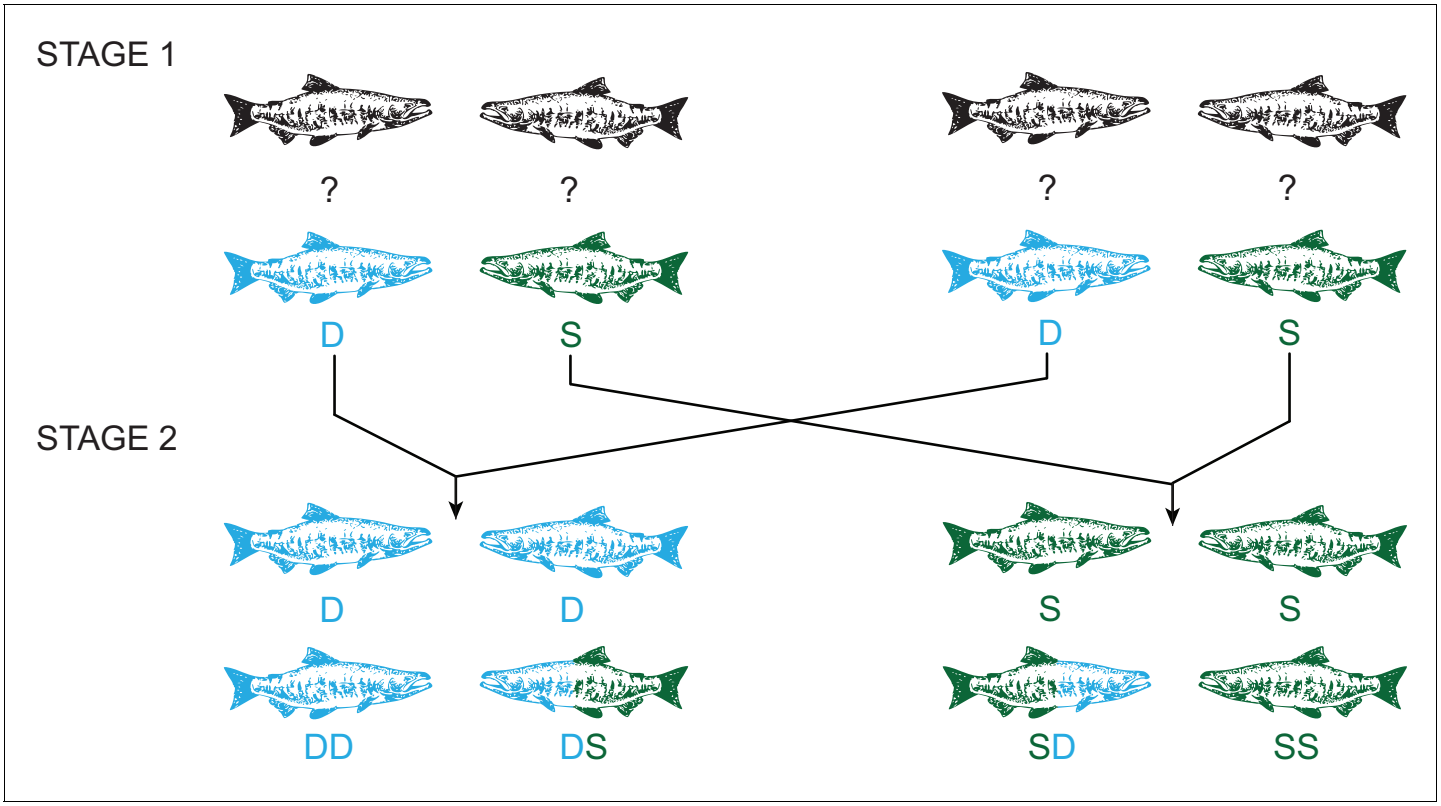

Figure 1. Experimental design using a two-stage social status manipulation in chinook salmon. For each trial, in stage 1, four males of unknown social status were used to form two pairs and the social hierarchy within each pairing was then determined, assigning one male as dominant (D) and the other subdominant (S). After 48 hr, ejaculates were collected from each male (D, S, D, S). In stage 2, we reformed pairs, putting males with the same social status together, and re-determined the social hierarchy within each pairing. Males either retained the same status, dominant (DD) or subdominant (SS) in both stages, or changed status in either direction, dominant to subdominant (DS) or subdominant to dominant (SD). After 48 hr, ejaculates were recollected from each male (DD, DS, SD, SS).

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closer to identifying the underlying molecular mechanism that enables plasticity of ejaculate performance in dynamic social environments.

Results

Social status and ejaculate quality

Subdominant (S) males had on average faster swimming sperm (Average Path Velocity, or VAP) than dominant (D) males. This difference was not significant when social status was initially determined in stage 1 (***Table 1***; ***Figure 2a***) but was significant for stage 2 (***Table 1***; ***Figure 2b***). Overall, there was considerable variation in sperm swimming speeds among males, accounted for by the random predictor 'male identity' that was significant in both stages (stage 1: $\chi^2_{(1)}$=105.11, p<0.001; stage 2: $\chi^2_{(1)}$=70.02, p<0.001). Additionally, sperm concentration was significantly higher in S than in D males in stage 1 (***Table 1***; ***Figure 3a***), but not stage 2 (***Table 1***; ***Figure 3b***). However, sperm concentration for males that remained subdominant (SS) was significantly higher than for those males that remained socially dominant (DD) in stage 2 (***Table 1***).

Ejaculate plasticity in response to social status change

There was a significant increase in mean VAP for males that changed from dominant to subdominant social status (DS; ***Table 2***; ***Figure 4***). Throughout the social status experiment, there were no other significant changes in either VAP or sperm concentration for males of the other social phenotypes (***Table 2***; ***Figure 4***). There was also a significant overall interaction effect between social phenotype

Table 1. Generalised linear mixed effects models (GLMM) to compare sperm velocity (VAP, μs^{-1}) and sperm concentration (cells/ml) among male chinook salmon of different social status (see ***Figure 1*** for experimental design).

In stage 1 of the experiment, dominant (D; n = 22) males were compared to subdominants (S; n = 22). In stage 2, separate models were run with the fixed parameter social status with either four levels (males that retained the same status DD (n = 10) and SS (n = 9), and males that changed status SD (n = 9) and DS [n = 10]), or two levels with data pooled together (D = D**D** + S**D** (n = 19), S = S**S** + D**S** (n = 19)). p-Values are calculated using Satterthwaite approximations to degrees of freedom and 95% confidence intervals were calculated using the Wald method. p-Values are adjusted for multiple testing where multiple pairwise comparisons are made using the Bonferroni method with significant values highlighted in bold.

Response variable	Stage	Parameters (fixed effects)	Estimate	95% CI	*p* Value
VAP	1	Intercept	152.9	135.3–170.4	
		D – S	7.4	−8.6–23.4	0.37
	2	Intercept	127.1	108.8–145.5	
		D – S	19.7	5.1–34.2	**0.01**
	2	Intercept	131.2	109.2–153.2	
		DD – SS	14.9	−6.5–36.5	0.18
		DD – DS	17.9	−2.7–38.5	0.09
		SD – DS	24.4	2.9–45.9	0.03
		SD – SS	21.5	−0.2–43.2	0.06
Sperm concentration	1	Intercept	6.0	5.81–6.22	
		D – S	0.2	0.01–0.39	**0.04**
	2	Intercept	5.9	5.72–6.21	
		D – S	0.2	−0.06–0.41	0.14
	2	Intercept	5.8	5.55–6.09	
		DD – SS	0.5	0.16–0.77	**0.003**
		DD – DS	0.1	−0.16–0.43	0.36
		SD – DS	−0.1	−0.44–0.18	0.42
		SD – SS	0.2	−0.12–0.52	0.21

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and experimental stage on VAP ($\chi^2_{(3)}$=11.8, p=0.008), with a significant interaction effect found only for males changing from dominant to subdominant status (DS; p=0.02, 95% CI = 2.9–34.9). We found no significant interaction effects between social phenotype and experimental stage on sperm concentration ($\chi^2_{(3)}$=3.0, p=0.385).

Seminal fluid effect on sperm velocity

Within each dyad, the social status of the rival male was a significant predictor of the difference in VAP between focal male's sperm incubated in their own seminal fluid and the focal male's sperm incubated in their rival's seminal fluid (***Table 3***). Seminal fluid from subdominant males increased the sperm swimming speed of sperm from dominant males, conversely, the seminal fluid from dominant males decreased sperm swimming speed of the sperm from subdominant males (***Figure 5***). However, rival's social status was no longer significant (***Table 3***) when the difference in VAP between the focal male control and rival male control was added as a fixed predictor to the model, for which a significant positive linear relationship was detected (***Table 3***), with sperm in the seminal fluid of a rival that had faster VAP increasing sperm velocity and sperm in the seminal fluid of a rival that had slower VAP decreasing sperm velocity relative to VAP in their own seminal fluid (***Figure 6***).

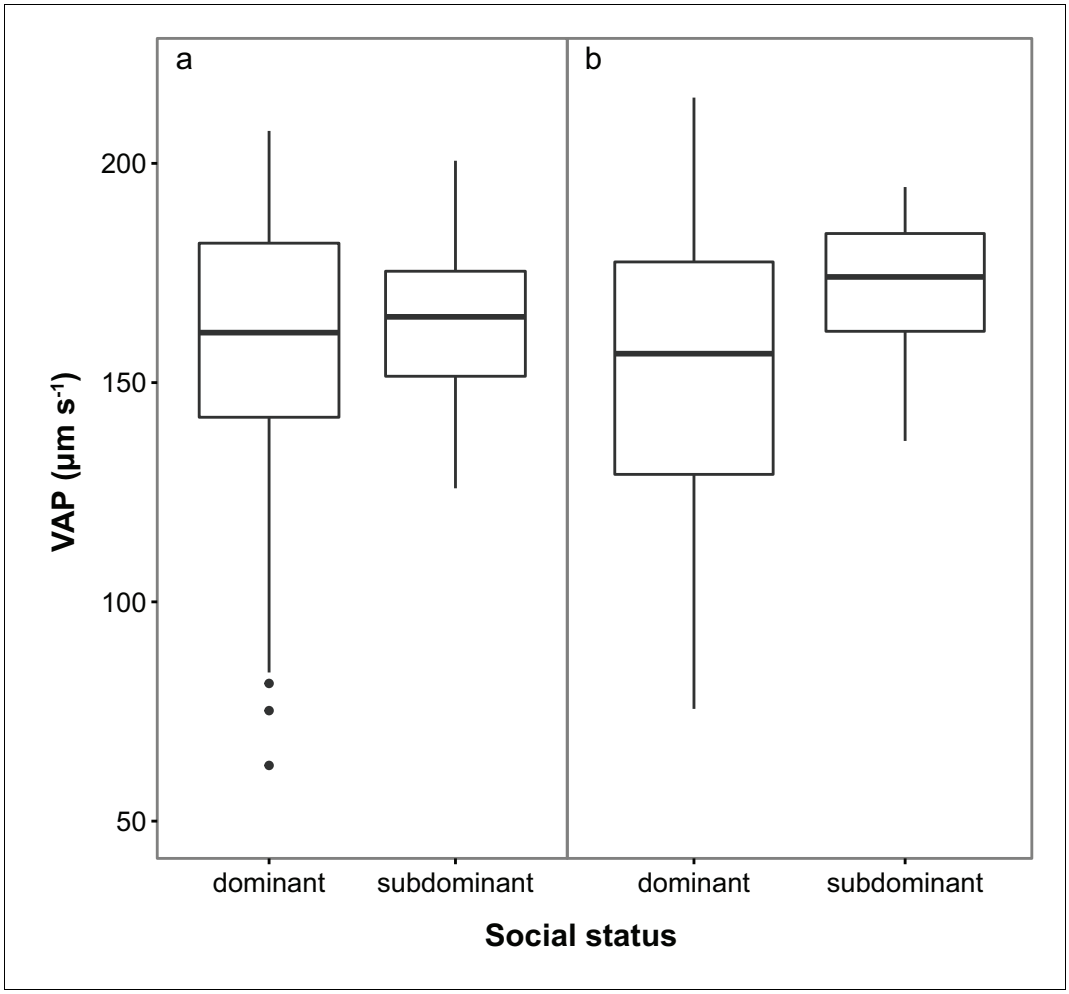

Figure 2. Sperm velocity (VAP in μm s^{-1}) in males of dominant (D) and subdominant (S) social status after a: the first social challenge (D, n = 22; S, n = 22) and b: the second social challenge (D, n = 19; S, n = 19). Boxplots display the median of each group with the 25th and 75th percentiles and whiskers extend to data within 1.5 x the inter-quartile range.

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The following source data and figure supplements are available for figure 2:

Source data 1. Source data for boxplot (***Figure 2a***).

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Source data 2. Source data for boxplot (***Figure 2b***).

DOI: https://doi.org/10.7554/eLife.28811.007

Figure supplement 1. Across all sperm samples collected in this study, Average Path Velocity (VAP) at 10 s post-activation was strongly correlated with Curvilinear Velocity (VCL; r = 0.85, p<0.000 l, n = 126).

DOI: https://doi.org/10.7554/eLife.28811.005

Figure supplement 1—source data 1. Source data for correlation analysis.

DOI: https://doi.org/10.7554/eLife.28811.008

In vitro fertilisation trials

Male social status was a significant predictor of the proportion of eggs sired (***Table 4***), with subdominant males siring a greater proportion (0.54 ± 0.08 95% CI, n = 21) than dominant males (0.46 ± 0.06 95% CI, n = 21). The social status of the seminal fluid donor when seminal fluid was swapped between males was also a significant predictor of the proportion of eggs sired (***Table 4***), with sperm incubated in the seminal fluid of subdominant males siring a greater proportion (0.6 ± 0.09 95% CI, n = 21) of eggs than sperm incubated in the seminal fluid of dominant males (0.4 ± 0.09 95% CI,

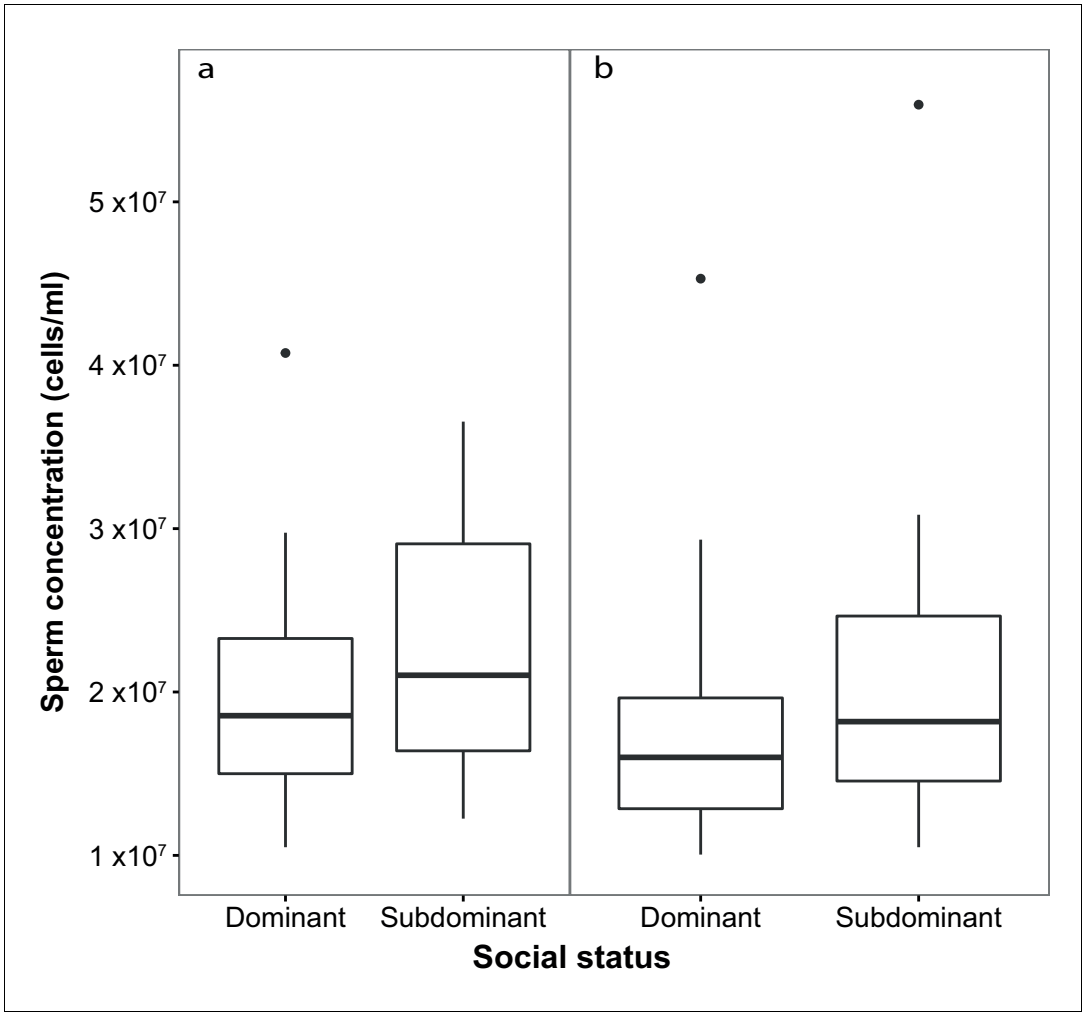

Figure 3. Sperm concentration (cells/ml) in males of dominant (D) and subdominant (S) social status after a: the first social challenge (D, n = 22; S, n = 22) and b: the second social challenge (D, n = 19; S, n = 19). Boxplots display the median of each group with the 25th and 75th percentiles and whiskers extend to data within 1.5 x the inter-quartile range.

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The following source data is available for figure 3:

Source data 1. Source data for boxplot (***Figure 3a***).

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Source data 2. Source data for boxplot (***Figure 3b***).

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n = 21). The difference in sperm velocity between competitors was also a significant predictor of the proportion of eggs sired in both unmanipulated (***Table 4***) and recombined ejaculate seminal fluid treatments (***Table 4***). The change in relative sperm velocity between males within the same male-male-female combinations across seminal fluid treatments was a significant predictor of the change in the proportion of eggs sired by that male's sperm across treatments (***Table 4***; ***Figure 7***).

Discussion

In this study, we experimentally manipulated social status to produce four social phenotypes with differing levels of sperm competition risk, and in accordance with sperm competition theory (***Parker, 1990***; ***Parker et al., 1997***; ***Parker, 1998***; ***Wedell et al., 2002***; ***Birkhead et al., 2009***; ***Parker and Pizzari, 2010***), found males with the highest risk of sperm competition produced

Table 2. Generalised linear mixed effects models (GLMM) to compare sperm velocity (VAP, μs^{-1}) and sperm concentration (cells/ml) in males of each social phenotype changing from stage 1 to stage 2 of the experiment.

The four social phenotypes are males that remained dominant (DD, n = 10) or subdominant (SS, n = 9) in both stages and males that changed status in either direction, subdominant to dominant (SD, n = 9) and dominant to subdominant (DS, n = 10). p-Values are calculated using Satterthwaite approximations to degrees of freedom and 95% confidence intervals were calculated using the Wald method. p-Values are adjusted for multiple testing using the Bonferroni method with significant values highlighted in bold.

Response variable	Social phenotype	Parameters (fixed effects)	Estimate	95% CI	p alue
VAP	DD	Intercept	109.1	88.9–129.2	
		Stage 1 – Stage 2	0.1	−14.1–14.4	0.9
	SD	Intercept	139.6	111.9–167.2	
		Stage 1 – Stage 2	−8.9	−19.5–1.5	0.1
	DS	Intercept	163.9	141.1–186.8	
		Stage 1 – Stage 2	17.2	5.4–29.1	**0.008**
	SS	Intercept	162.5	147.1–177.9	
		Stage 1 – Stage 2	−2.3	−12.0–7.4	0.6
Sperm concentration	DD	Intercept	5.6	5.34–5.97	
		Stage 1 – Stage 2	−0.2	−0.39–0.06	0.2
	SD	Intercept	6.4	6.12–6.68	
		Stage 1 – Stage 2	−0.2	−0.48–0.002	0.05
	DS	Intercept	6.1	5.56–6.58	
		Stage 1 – Stage 2	−0.1	−0.34–0.15	0.4
	SS	Intercept	6.4	6.09–6.61	
		Stage 1 – Stage 2	0.1	−0.17–0.35	0.5

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ejaculates with both higher sperm concentration and faster swimming sperm. We also found males can make rapid adjustments to sperm velocity in a strategic response to changes in social position that signal increased sperm competition risk. While seminal fluid is often *implicated* to harbour the unknown mechanism behind plastic sperm performance in vertebrates (***Perry et al., 2013***; ***Fitzpatrick and Lüpold, 2014***), our combined results for the first time, unequivocally demonstrate that seminal fluid acts as a mediator of rapid strategic adjustment to sperm velocity. Furthermore, we demonstrate strategic adjustments of sperm velocity mediated by seminal fluid directly impact male fitness, highlighting the adaptive significance of plastic ejaculate performance.

Sperm competition theory predicts that males should strategically adjust ejaculates in response to changing sperm competition risk (***Wedell et al., 2002***; ***Parker and Pizzari, 2010***). In chinook salmon, relative sperm velocity among males is the primary determinant of fertilisation success (***Evans et al., 2013***; ***Rosengrave et al., 2016***). We show males forced to change from dominant to subdominant social status, and therefore exposed to increased sperm competition risk, responded by increasing the quality of their ejaculate, in this case sperm velocity, within 48 hr (***Figure 4***). While we predict that males forced to change from subdominant to dominant social status, therefore exposed to decreased sperm competition risk, would respond by decreasing their ejaculate quality, we did not see a significant change in sperm velocity for these males. However, subdominant males that later became dominant had a relatively low mean sperm velocity that was more similar to dominant males than those from the other subdominant phenotype in the first stage of the experiment (***Figure 4***). In this case, these subdominant males may have attempted to adopt a guarding tactic even after losing in the first social challenge, as males that lose contests can either sneak or fight for dominance elsewhere (***Esteve, 2005***).

Males should also strategically adjust sperm concentration in response to changing sperm competition risk (***Wedell et al., 2002***; ***Parker and Pizzari, 2010***). Accordingly, we found subdominant males produced ejaculates with greater sperm concentration than dominant males. However, our

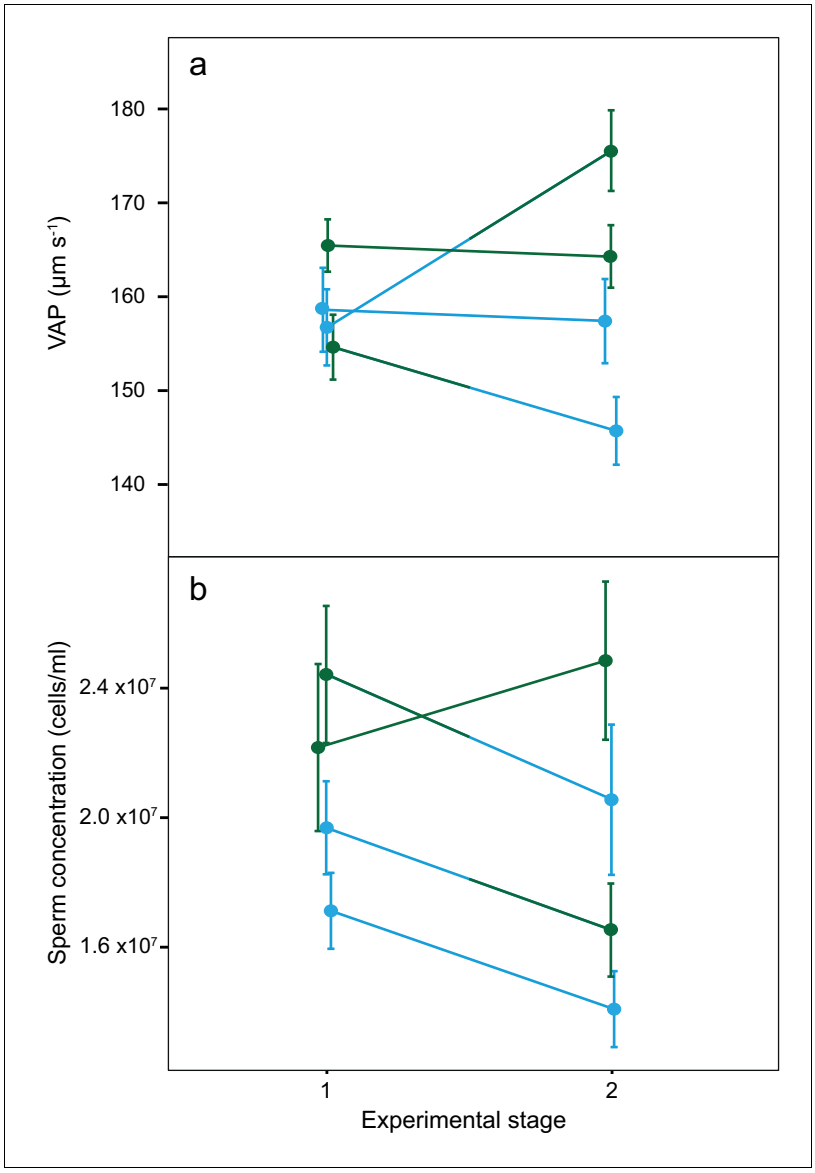

Figure 4. Average sperm velocity (VAP, µm s^{-1};±s.e.m.) and average sperm concentration (cells/ml;±s.e.m.) in males of the four social phenotypes after each stage of a social status manipulation experiment in chinook salmon. Blue colour denotes males dominant in both stages (DD, n = 10), green colour denotes males subdominant in both stages (SS, n = 9), a change from blue to green colour denotes males that changed from dominant to subdominant status (DS, n = 10) and a change from green to blue colour denotes males that changed from subdominant to dominant status (SD n = 9). The change in VAP for DS males was statistically significant.

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The following source data is available for figure 4:

Source data 1. Source data for ***Figure 4a***.

DOI: https://doi.org/10.7554/eLife.28811.014

Source data 2. Source data for ***Figure 4b***.

DOI: https://doi.org/10.7554/eLife.28811.015

results show that there was no significant increase in sperm concentration for any of the social phenotypes over a 48-hr period. The exact time taken for spermatogenesis in salmonids is unknown; however, the process almost certainly takes more than 48 hr (***Billard, 1983a***; ***Billard, 1983b***; ***Schulz et al., 2010***). Therefore, these results suggest that the observed changes in sperm velocity

Table 3. Generalised linear mixed effects models (GLMM) predicting the change in sperm velocity (VAP, μs^{-1}) observed in the focal male's sperm when incubated in either their own seminal fluid or the seminal fluid of their rival male in that dyad, using the social status of the rival male and the relative VAP between sperm from focal and rival males as measured in their own seminal fluid (n = 42 males in 39 dyads).

p-Values are calculated using Satterthwaite approximations to degrees of freedom and 95% confidence intervals were calculated using the Wald method. Significant values are highlighted in bold.

Response variable	Model	Parameters (fixed effects)	Estimate	95% CI	p-Value
Change in VAP	1	Intercept	−24.4	−41.8–−7.0	
		Rival's Social Status	31.4	15.1–47.7	**0.0003**
	2	Intercept	−0.64		
		Rival's Social Status	0.44	−0.7–1.6	0.465
		Relative VAP	0.05	0.04–0.07	**<0.0001**

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are mediated by a component of the ejaculate that modifies the competitiveness of existing sperm, rather than simply via the production of new sperm.

Our results clearly demonstrate the observed plasticity of sperm velocity in chinook salmon, a key determinant of fertilisation success in several vertebrate species (***Birkhead et al., 1999***; ***Malo et al., 2005***; ***Gasparini et al., 2010***; ***Boschetto et al., 2011***) including salmonids (***Gage et al., 2004***; ***Liljedal et al., 2008***; ***Evans et al., 2013***; ***Egeland et al., 2015***; ***Rosengrave et al., 2016***), is mediated by seminal fluid. We found sperm from the same male, when incubated in seminal fluid from different males, had significantly different sperm velocities, and the direction of this effect could be predicted by social status. For example, when sperm from dominant males were incubated in seminal fluid from subdominant males we found that on average their sperm velocity increased

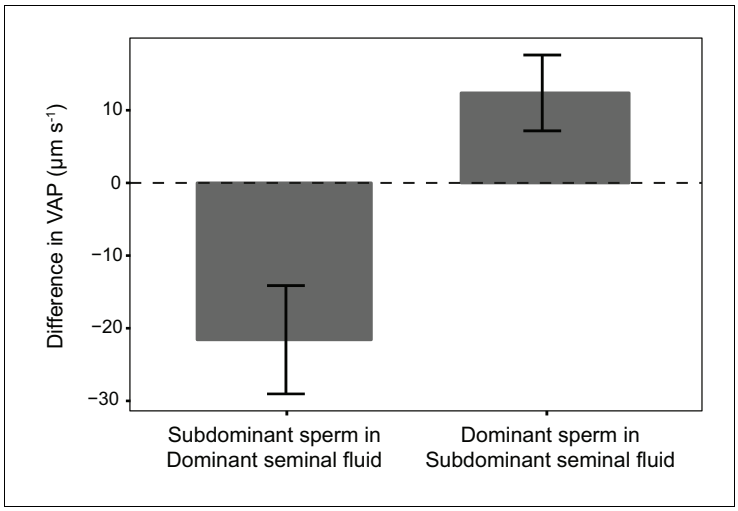

Figure 5. Average difference in sperm velocity (VAP, $\mu m\ s^{-1}$;±s.e.m.) between sperm incubated in their own seminal fluid and incubated in the seminal fluid of their rival in each dyad of a social status manipulation experiment in chinook salmon (n = 42 males in 39 dyads). Seminal fluid from dominant rival males on average decreased VAP of sperm from subdominant males. In contrast, seminal fluid from rival subdominant males on average increased VAP of sperm from dominant males. Social status was a significant predictor of the difference in sperm velocity between sperm incubated in their own seminal fluid and incubated in the seminal fluid of their rival.

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The following source data is available for figure 5:

Source data 1. Source data for ***Figure 5***.

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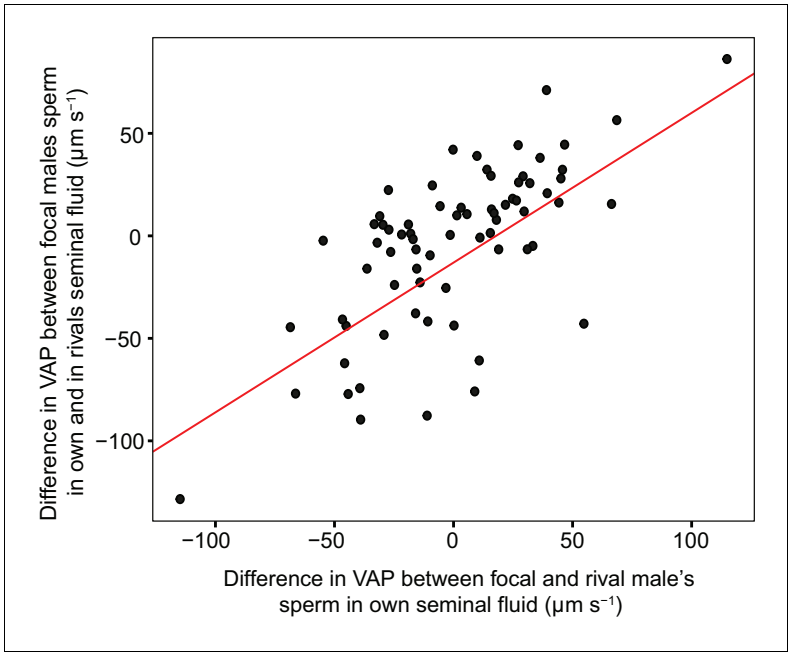

Figure 6. Significant linear relationship between the difference in sperm velocity (VAP, μm s^{-1}), between sperm incubated in their own seminal fluid and incubated in the seminal fluid of their rival, and the difference in VAP between sperm from the males in each pairing incubated in their own seminal fluid for each dyad of a social status manipulation experiment in chinook salmon (n = 42 males in 39 dyads). Incubating sperm in the seminal fluid of a rival with faster VAP generally results in an increase in that male's sperm velocity. Likewise, incubating sperm in the seminal fluid of a rival with slower VAP generally results in a decrease in that male's sperm velocity. Raw data is displayed for ease of interpretation, data analysis required transformation (refer to Materials and methods: Statistical analyses and supplementary material for details).

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compared to the baseline measures in their own seminal fluid, and found the opposite effect when sperm from subdominant males were incubated in seminal fluid from dominant males (***Figure 5***). Contrary to ***Cornwallis and O'Connor, 2009***, for which seminal fluid from higher quality ejaculates decreased the velocity of sperm from lower quality ejaculates in fowl, our findings are consistent with the prediction that seminal fluid from ejaculates with faster swimming sperm will enhance the speed of sperm from ejaculates with slower sperm. The disparity between our findings and those in fowl (***Cornwallis and O'Connor, 2009***) possibly reflect differences in the reproductive biology of these species; including internal and external modes of fertilisation and differences in the structure and formation of social hierarchies and associated sperm competition risk.

Ejaculate allocation in fowl is also influenced by factors other than sperm competition risk, including female quality and the probability of future mating opportunities (***Pizzari et al., 2003***; ***Cornwallis and Birkhead, 2006***; ***Cornwallis and Birkhead, 2007a***; ***Cornwallis and Birkhead, 2007b***); whether such factors influence ejaculate allocation strategies in salmonids is unknown. It is also possible that seminal fluid in fowl has evolved to interact with sperm from rivals, as observed in some insect species (***den Boer et al., 2010***) and reported for the grass goby (*Zosterisessor ophiocephalus*) (***Locatello et al., 2013***). Fertilisation occurs rapidly in salmonids, with the majority of eggs fertilised within 10 s post ejaculation (***Hoysak and Liley, 2001***; ***Liley et al., 2002***; ***Yeates et al., 2007***). Such rapid time frames may allow for little interaction between seminal fluid and sperm from different males during spawning. This is supported by research using Arctic charr that found the activation of sperm with a solution containing seminal fluid from another male had no effect on sperm velocity (***Rudolfsen et al., 2015***). However, a recent experiment that separated and recombined ejaculates from precocious chinook salmon males (obligate sneakers) and adult hooknose males report similar results to those found in the grass goby, with seminal fluid from precocious males significantly decreasing the velocity of hooknose male sperm (***Lewis and Pitcher, 2017***). Our results

Table 4. Generalised linear mixed effects models (GLMM) predicting the fertilisation success of male chinook salmon in sperm competition trials using two males and one female.

Trials were conducted using two seminal fluid (SF) treatments, either unmanipulated milt, or recombined ejaculates for which the sperm for both competitors were recombined with the seminal fluid of their rival. Sperm concentration was controlled so that the same number of sperm were used for each male. The first models used the social status of each male to predict the proportion of offspring sired (n = 20). The second models used the relative sperm velocity (VAP, μm s^{-1}) between competitors to predict the difference in offspring sired (n = 20). The final model shows that the change in relative sperm velocity between males within the same male-male-female combinations across SF treatments was a significant predictor of the change in the proportion of eggs sired by that male's sperm across SF treatments (n = 20). p-Values are calculated using Satterthwaite approximations to degrees of freedom and 95% confidence intervals were calculated using the Wald method. Significant values are highlighted in bold.

Response variable	SF treatment	Parameters (fixed effects)	Estimate	95%	P value
Proportion of offspring sired	Unmanipulated	Intercept Social status	−0.38 1.11	0.63–1.58	**<0.0001**
	Recombined	Intercept SF social status	−3.24 6.23	4.7–7.7	**<0.0001**
Difference in number of offspring sired between males	Unmanipulated	Intercept Relative sperm velocity	$-1.49e^{03}$ $1.44e^{-01}$	0.06–0.23	**0.003**
	Recombined	Intercept Relative sperm velocity	$3.72e^{03}$ 0.13	0.05–0.21	**0.003**
Difference in proportion of eggs sired across SF treatments	NA	Intercept Difference in relative sperm velocity across SF treatments	−56.39 0.006	$3.5e^{-03}$– $7.6e^{-03}$	**0.0001**

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The following source data available for Table 4:

Source data 1. Source data for GLMM models predicting the fertilisation success of male chinook salmon in sperm competition trials.

This Excel file contains data on the proportion of eggs sired by each male and the social status of those males. The data is presented in two tabs, the first for the unmanipulated milt and the second for the recombined ejaculate seminal fluid treatments.

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Source data 2. Source data for GLMM models predicting the fertilisation success of male chinook salmon in sperm competition trials.

This Excel file contains data on the diffence in the number of eggs sired between males in each sperm competition trial and the relative sperm velocity of those males. The data is presented in two tabs, the first for the unmanipulated milt and the second for the recombined ejaculate seminal fluid treatments.

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suggest chinook salmon seminal fluid has not evolved a targeted effect on sperm from males adopting a different tactic within the same age/size class, as regardless of social status, males that have faster recorded sperm velocities produced seminal fluid that increases the velocity of sperm from other males with slower speeds, and likewise males with slower sperm velocity produced seminal fluid that decreases the velocity of sperm from males with faster speeds (***Figure 6***).

In addition to demonstrating that seminal fluid influences sperm competitiveness, our *in vitro* sperm competition trials show the influence seminal fluid has on sperm velocity translates to having an effect on male fitness. We measured the fertilisation success within the same male x male x female combinations across trials, and compared those males across unmanipulated and recombined ejaculate treatments, finding changes in the relative sperm velocity between competitors were significantly correlated with the change in the proportion of eggs sired by each male (***Figure 7***). That is, the change in sperm velocity due to the seminal fluid in which sperm were incubated had a significant influence on the proportion of eggs sired by those sperm, in some cases completely reversing the 'winner' of sperm competition within the same male-female group. We now need further investigation to determine the component of seminal fluid that is strategically adjusted by males in response to sperm competition risk.

Previous studies have found that natural variation in several seminal fluid metrics was not correlated with sperm velocity in chinook salmon, including pH, osmolality and ion composition (***Rosengrave et al., 2009a***; ***Flannery et al., 2013***). It is possible that seminal fluid contains different levels of available nutrients therefore fuelling differential energy production in sperm. In the short

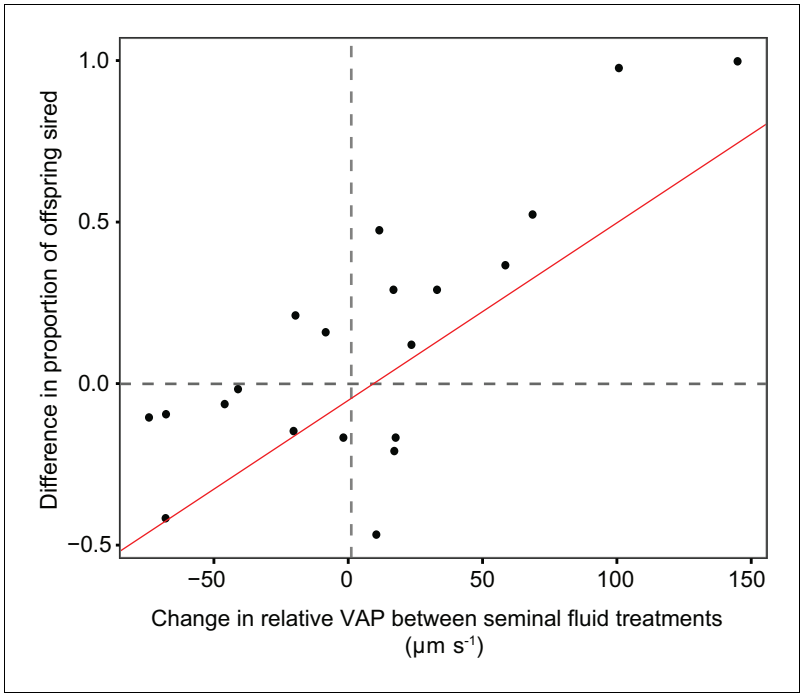

Figure 7. Statistically significant relationship between the difference in the proportion of eggs sired by the focal male in each triad from sperm competition trials using chinook salmon (n = 20) when that male's sperm were either incubated in their own or their rival's seminal fluid, and the difference in relative sperm velocity (VAP, μm s^{-1}) between males in each pair when sperm were either incubated in their own or their rival's seminal fluid. The relationship shows that change in fertilisation success across seminal fluid treatments is correlated with the change in relative sperm velocity between competing males in each seminal fluid treatment.

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The following source data is available for figure 7:

Source data 1. Source data for ***Figure 7***.

DOI: https://doi.org/10.7554/eLife.28811.022

term following activation of motility in salmonids, sperm utilise ATP as the energy source for flagellar movement (***Christen et al., 1987***) using both stored ATP reserves and increasing ATP production significantly via aerobic respiration (***Lahnsteiner et al., 1993***, ***Lahnsteiner et al., 1999***). Sperm ATP levels have been positively correlated with sperm velocity (***Lahnsteiner et al., 1998***; ***Bencic et al., 1999***; ***Burness et al., 2004***) and fertilisation success (***Zilli et al., 2004***; ***Vladić et al., 2010***) in external fertilisers. Exposure to different levels of exogenous nutrients in seminal fluid while sperm are immotile in the testis may influence energy metabolism, for example altering available energy reserves or stored nutrient reserves, influencing sperm velocity post activation (***Lahnsteiner et al., 1999***). Alternatively, seminal fluid may contain peptide or RNA signalling molecules, that alter sperm behaviour. For example, chemotaxis in several marine invertebrates is controlled by signalling pathways that are initiated by chemoattractant peptides released by ova (***Kaupp et al., 2003***; ***Darszon et al., 2008***; ***Evans and Sherman, 2013***). Evidence is also accruing that proteins and RNAs in seminal fluid exosomes may play critical roles in regulating sperm development and fertilisation (***Vojtech et al., 2014***; ***Jodar et al., 2016***).

Several Seminal Fluid Proteins (SFPs) have been associated with sperm velocity in vertebrate species (***Lahnsteiner et al., 1996***, ***1998***; ***Poiani, 2006***) and are therefore likely candidates for modifying rapid adjustment of sperm velocity (***Simmons and Fitzpatrick, 2012***). Differences in SFP composition have been documented among males adopting different reproductive tactics in externally fertilising fish (***Scaggiante et al., 1999***; ***Gombar et al., 2017***). Additionally, a growing body of empirical work has demonstrated that males can tailor SFP composition in response to sperm competition risk (***Wigby et al., 2009***; ***Fedorka et al., 2011***; ***Ramm et al., 2015***; ***Simmons and Lovegrove, 2017***)

and the mating status of females (**Sirot et al., 2011**). The role of SFPs in sperm competition, with the exception for some insect species (**den Boer et al., 2010**; **Avila et al., 2011**) and specific proteins in mammals (**Ramm et al., 2008**), is generally poorly understood. While the activity of SFPs associated with energy metabolism and respiration have been correlated with sperm velocity in a Cyprinid species (**Lahnsteiner et al., 1996**) and rainbow trout (*O. mykiss*) (**Lahnsteiner et al., 1998**), total protein concentration as well as the activity of lactate dehydrogenase, anti-trypsin and superoxide dismutase enzymes were not correlated with sperm velocity in chinook salmon (**Flannery et al., 2013**). However, these SFPs represents only a small fraction of the enzymatic activity likely to occur in fish seminal fluid (**Gombar et al., 2017**; **Nynca et al., 2014**). The critical next step in determining the molecular mechanism(s) involved will be to link variation in seminal fluid components to sperm velocity, and confirm these results experimentally.

In conclusion, as predicted by sperm competition theory (**Parker, 1990**; **Parker et al., 1997**; **Parker, 1998**; **Wedell et al., 2002**; **Birkhead et al., 2009**; **Parker and Pizzari, 2010**), we find male chinook salmon can make rapid adjustment to sperm velocity in response to social cues that signal changing sperm competition risk and such changes have a significant impact on the outcome of sperm competition and therefore male fitness. We further demonstrate that seminal fluid, even in a species with external fertilisation, plays a key role in mediating the strategic rapid adjustment of sperm velocity and for the first time provide strong evidence the mechanism behind plasticity in sperm velocity lies within the non-sperm component of the ejaculate. Our results support plastic adjustment of ejaculate quality in response to changing sperm competition risk is an effective evolutionary strategy in systems with dynamic social environments and we show seminal fluid mediates such adjustments.

Materials and methods

Study species and maintenance

Wild chinook salmon were caught during their annual spawning runs in a trap located on the Kaiapoi River, a tributary of the Waimakariri River system, Canterbury, New Zealand (**Unwin et al., 2000**). We studied a total of 17 sexually mature 3-year-old females and 44 sexually mature 3-year-old 'hook-nose' males captured between 27 April and 30 May in 2013, 2014 and 2015. Sample size was informed by related empirical research in this system (**Rosengrave et al., 2008**, **2009a**). Fish were individually tagged and maintained in a natural river-water raceway (12.5–13˚C) at a hatchery (Salmon Smolt NZ, Canterbury, New Zealand) using standard husbandry procedures. All animals were collected and maintained according to the standards of the Animal Ethics Committee for the University of Otago, New Zealand.

Manipulation of male social status

A total of 11 social status manipulation trials were conducted each using four males (n = 44; **Figure 1**). On day 1, two male dyads were formed pairing males of similar size (average fork length = 71.5 cm, 95% CI = 70.2–72.9 cm, n = 44). Each dyad was then placed in a sectioned off part of a river-water raceway (approx. 2.5 m x 2 m x 1 m). Social interactions between the two fish in each dyad were observed for the first day using a series of 10 min under-water video recordings (GoPro Hero 3), one taken each hour over a 5-hr period, with the first recording starting 15 min after introducing fish to the raceway. Male dominance was then determined by calculating a Dominance Index (DI; **Winberg et al., 1991**; **Bailey et al., 2000**; see *Behavioural observations*) using the number of aggressive interactions between males. The male with the higher DI was ranked as dominant (D) and the male with the lower DI as subdominant (S, stage 1 - **Figure 1**). On day 2, male dyads were left undisturbed and male social status within each dyad established on day one typically remained unchanged (**Table 5**). On day 3, male dyads were re-formed placing dominant with dominant and subdominant with subdominant, and a new social hierarchy developed with male social status assigned to each male as described for day one. This forced one fish of each original dyad to change his social status (DS or SD), while the other retained their original status (DD or SS, stage 2 - **Figure 1**). On day 4, the male dyads were left undisturbed, and the experiment was complete on day 5. We determined social status after all the social challenges except in one case where no interaction between males was recorded in the second stage and thus these individuals were excluded from

Table 5. The Dominance Index (DI) of the Dominant (D) and Subdominant (S) males in 11 pairings (6 in stage 1 and 5 in stage 2).

In 2013, behavioural observations were conducted twice for each pair, on the day the pair was formed (as in other years) and the next day as a means to assess the stability of social hierarchies. We found that in 10 out of 11 pairs the status of males determined on the first day did not change from on the second day.

Social status		D	D	S	S
Pair	Stage	Day 1	Day 2	Day 1	Day 2
1	1	0.844	0.739	0.155	0.26
2	1	0.8	0.75	0.19	0.25
3	2	0.829	0.857	0.17	0.14
4	2	1	0.93	0	0.06
5	1	0.98	1	0.01	0
6	1	0.96	0.89	0.03	0.1
7	2	0.82	0.15	0.2	0.8
8	1	0.97	1	0.03	0
9	1	1	1	0	0
10	2	0.85	1	0.15	0
11	2	1	1	0	0

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further analyses. A further four males were excluded from analyses due to males escaping from the raceway in the second stage of the experiment, giving a total sample sizes n = 44 in stage 1 and n = 38 in stage 2.

Behavioural observations

Dominance Index (DI) was calculated using the following equation:

$DI = Agg^{+} / (Agg^{+}+Agg^{-})$,

where Agg^{+} represents the total number of aggressive acts performed and Agg^{-} the total number of aggressive acts received by the individual (***Zilli et al., 2004***; ***Bailey et al., 2000***). Aggressive acts were scored using the following criteria:

Charge

Makes a rapid movement towards the other male.

Chase

Continual movement towards the other male with that male actively moving away from aggressor. Each lap around the enclosure from the point where the chase was initiated was scored as one chase, such that continual chasing without pause was scored repeatedly.

Bite

Bites the body of the other male with full gape.

Nip/Nudge

Bites the tail fin of the other male or nudges the other male with a closed mouth.

Measurement of ejaculate quality

Ejaculates were obtained from males by gently applying pressure to the abdomen, taking care to avoid contaminating samples, and were held at 4°C for up to 4 hr. We depleted the ejaculate reserves of each male before the experiment so ejaculates collected later were produced during each 48-hr period. We collected ejaculates in a random order on day 3 at the end of stage 1 and

after social status was manipulated on day 5 at the end of stage 2 so samples were collected 48 hr after social status was established in each stage.

Sperm velocity measurements were performed in a random order and blind to the social status of each male. We measured sperm swimming speed twice for each male at 10 s post-activation using a CEROS sperm tracker (v 1.2, Hamilton-Thorne Research, Beverly, MA). Approximately 1 µl of milt was activated with river water or ovarian fluid (diluted to 50% with river water) onto a 20 µl Leja slide (Leja Products B.V., Nieuw-Vennep, The Netherlands) on a temperature-controlled stage cooler (TS-4 Thermal Microscope Stage, Physitemp) set to 12.5˚C to match the natural spawning water temperature. We used average path velocity (VAP, µm s^{-1}) as our measure of sperm swimming speed which estimates the average velocity of a sperm cell for 0.5 s over a smoothed path (***Rosengrave et al., 2008***, ***2009a***, ***2016***; ***Figure 2—figure supplement 1***). Sperm concentration (sperm/ml) was determined using a Neubauer haemocytometer.

Manipulation of ejaculates

To determine the relative roles of sperm and seminal fluid on sperm velocity we centrifugally separated and remixed sperm and seminal fluid of each male with those from the other male in each dyad (n = 42 males in 39 dyads). To prepare recombined ejaculates, milt was centrifuged in 1.5 ml tubes at 4˚C, 300 g for 10 min to separate sperm cells from seminal fluid. The seminal fluid was then transferred into a new tube after which 500 µl of artificial seminal fluid (80 mM NaCl, 40 mM KCl, 1 mM $CaCl_2$, 20 mM Tris-HCl) was added to the sperm cells and this was centrifuged again at 4˚C, 300 g for 10 min to wash any remaining seminal fluid from the sperm cells. The artificial seminal fluid was then discarded and recombined ejaculates were prepared using 10 µl of sperm resuspended in 90 µl of seminal fluid from the same male (control) or seminal fluid from their rival, incubated at 12˚C for 20 min.

In vitro fertilisation trials

In 2014 and 2015, at both stages of the social status manipulation trials (***Figure 1***) we conducted a total of 21 replicated *in vitro* fertilisation trials to determine the effects of ejaculate recombination (seminal fluid) on male fertilisation success. This involved 24 individual males and 17 females in which sperm from the dominant and subdominant male in each dyad competed to fertilise a female's eggs. For each trial, we performed two seminal fluid treatments, using either unmanipulated or recombined ejaculates, in addition to non-competitive controls using sperm from each of the males individually. Haphazardly chosen female fish were killed with a stroke to the head, and their egg batch was expelled through an incision in the abdomen, into a clean bowl. Ovarian fluid was collected by carefully pipetting from each egg batch. Sperm density was adjusted prior to each fertilisation trial so that approximately the same number of sperm per male (10^7 spermatozoa) were used in each trial.

For each trial, we placed approximately 100 unfertilised ova from the focal female in a dry 2 l plastic beaker, then added ejaculate samples from each male simultaneously by injecting them separately into a steady stream of raceway water (250 ml at 12.5–13 ˚C). This technique simulated natural spawning conditions by facilitating the rapid mixing of eggs with sperm from both males (***Rosengrave et al., 2016***). We added the ejaculate samples separately into the water to ensure the spermatozoa were activated before the ejaculate samples came into contact, minimising any effects of each male's seminal fluid on the other male's sperm function. The eggs were allowed to sit for 5 min undisturbed until water hardened and were then gently transferred to heath rack trays (12.5–13˚C). We randomly sampled 24 alevins from each replicate fertilisation trial (40 days post fertilisation), placing them in 99% ethanol for DNA extraction and microsatellite genotyping to assess paternity.

DNA extraction, microsatellite amplification and genotyping protocols

To assess paternity share for the males in each sperm competition trial, DNA was extracted from a fin clip for both adult males, the female and 24 offspring from each trial using Chelex100 resin (***Walsh et al., 1991***). Three microsatellite loci (Ots 100, Ots 101, Oki 3a; ***Table 6***) were then amplified in a multiplex PCR and used to determine paternity by manually matching alleles between offspring, mother and either potential sire. A fourth locus (Ots 104; ***Table 6***) was amplified separately

Table 6. Microsatellite primers used to determine paternity.
Primers Ots 100, Ots 101 and Oki 3a were amplified in a multiplex reaction, Ots 104 was amplified singly using a touchdown protocol. Letter at 5′ end indicates fluorescent label: p=Pet (red), F = Fam (blue), N = Ned (yellow), V = Vic (green).

Primer		Primer sequence 5′–3′	Master mix	PCR	Source
Ots 100	F	P-tga-aca-tga-gct-gtg-tga-g	Multiplex	Multiplex	***Nelson and Beacham (1999)***
	R	P-acg-gac-gtg-cca-gtg-ag			
Ots 101	F	F-acg-tct-gac-ttc-aat-tgg-t	Multiplex	Multiplex	***Small et al. (1998)***
	R	F-tat-taa-tcc-tcc-aac-cca-g			
Oki 3a	F	N-tgt-gct-ata-ggc-tga-atg-tgc	Multiplex	Multiplex	Unpublished, See, ***Kinnison et al. (2002)***
	R	N-aac-aca-ggc-atc-ccc-act-aa			
Ots 104	F	V-gca-ctg-tat-cca-cca-tga	Single	Touchdown	***Nelson and Beacham (1999)***
	R	V-gta-gga-gtt-tca-ttt-gaa-tc			

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using a touchdown PCR protocol and employed when three loci were insufficient to determine paternity without certainty. The genotype of each offspring was always consistent with the expected genotype based on the alleles for the potential sires, i.e. in no offspring did we record unique alleles present for both potential sires.

Multiplex PCRs were run in 10 µL volume reactions and included the following reagents: 1x PCR buffer (Bioline), 2 mM $MgCl_2$, 0.3 mM dNTPs, 0.4 µM forward and reverse Ots 101 primers, 0.2 µM forward and reverse Ots 100 and Oki 3a primers, 0.5 U of Bioline Taq DNA polymerase, and 0.5 µL of DNA. The thermal cycling conditions for the multiplex protocol were: 12 min at 95˚C followed by 10 cycles of 15 s at 94˚C, 30 s at 60˚C, and 30 s at 72˚C, followed by 30 cycles of 15 s at 89˚C, 30 s at 60˚C, 30 s at 72˚C, and a final extension period of 10 min at 72˚C.

PCRs for amplification of Ots 104 were run in 10 µL volume reactions and included the following reagents: 1x PCR buffer (Bioline), 2 mM $MgCl_2$, 0.3 mM dNTPs, 0.5 µM forward and reverse Ots 104 primers, 0.5 U of Bioline Taq DNA polymerase, and 0.5 µL of DNA. The thermal cycling conditions for the touchdown protocol were: 2 min at 95˚C followed by 10 cycles of 30 s at 95˚C, 45 s at Ta˚C, and 30 s at 72˚C, where Ta starts at 55˚C and drops by 0.5˚C each cycle (last cycle should be 50.5˚C), followed by 20 cycles of 30 s at 95˚C, 45 s at 50˚C, 30 s at 72˚C, and a final extension period of 10 min at 72˚C.

PCR samples were genotyped by adding 0.5 µL PCR product to 12 µL HiDi formamide and 0.3 µL Genescan LIZ500 size standard (Applied Biosystems) then run on an ABI3130 × 1 Genetic Analyser (Applied Biosystems). Results were visualised using GeneMarker v 2.2 (SoftGenetics, RRID:SCR_015661) and alleles were scored manually.

Statistical analyses

All statistical analyses were performed using R v 3.1.3 (***R Core Team, 2016***; RRID:SCR_001905). To compare changes in ejaculate quality (sperm velocity (VAP) or sperm concentration) between D and S males, generalised linear mixed effects models (GLMM) were fitted using the package 'lme4' (***Bates et al., 2015***; RRID:SCR_015654). GLMMs using a Gaussian error distribution were fitted using VAP as the response variable, while GLMMs with a Poisson error distribution were fitted using sperm concentration as the response variable. Each GLMM used male social status as a fixed predictor, for stage 1 two levels comparing D and S; and for stage 2, separate models were run with either two levels comparing D and S males with data pooled together (D = D**D** + S**D** and S = S**S** + D**S**), or four levels (males that retained the same status DD and SS, and males that changed status SD and DS). Models with VAP as the response variable used both replicate measurements for each male and included male identity as a random predictor to account for repeated measures.

To test whether males that change social status adjust ejaculate quality, we compared both VAP (GLMMs using a Gaussian error distribution) and sperm concentration (GLMMs with a Poisson error distribution) in the same males across the two stages of the experiment. Four separate models were run for each of the response variables, separately comparing males in each of the four social

phenotypes (DD, DS, SD, SS) and each model used experimental stage (factor with two levels) as a fixed predictor. Additionally, we used an alternative analysis for each of the response variables to test for an interaction effect between social status and experimental stage, both models used social status (factor with four levels; DD, DS, SD and SS), experimental stage (factor with two levels) and the interaction between social status and experimental stage as fixed predictors. Male identity was included as a random predictor to account for repeated measures from the same male.

A linear mixed effects model (GLMM) was fit using the difference in VAP between focal male's sperm recombined with his own seminal fluid and focal male's sperm recombined with his rival male's seminal fluid as the response variable, with difference in VAP between focal male's sperm recombined with his own seminal fluid and rival male's sperm recombined with his own seminal fluid, and social status of rival's seminal fluid as fixed predictors. To fulfil the model's assumption of normality a cube-root transformation was performed on the response variable. We used the random predictors focal male identity, rival male identity and each pairing to account for repeated measures. All VAP measures used were those activated in river water, not ovarian fluid, to avoid female effects on sperm velocity (***Rosengrave et al., 2009b***, ***Rosengrave et al., 2016***) that could mask the influence of seminal fluid.

To assess the importance of sperm velocity as a predictor of fertilisation success, we used a GLMM that was fit using the difference in the number of offspring sired between the focal and rival male in each trial as the response variable, with the relative sperm velocity between males as a fixed predictor. To assess social status as a predictor of fertilisation success we used a binomial GLMM that was fit using the proportion of offspring sired by each male as the response variable, with male social status as a fixed predictor in unmanipulated milt trials and the social status of seminal fluid donor as a fixed predictor in swapped seminal fluid trials. In order to assess the influence of seminal fluid on male fertilisation success, we used a GLMM that was fit using the change in the proportion of eggs sired by each focal male across seminal fluid treatments (within the same triad, i.e. within the same male-male-female combination) as the response variable with the change in relative sperm velocity across treatments used as a fixed predictor. For all above models, we used the random predictors focal male identity, rival male identity, female identity and each unique triad to control for repeated measures. We tested for repeatability of replicate trials conducted for each triad (supplementary material: *Statistical analysis and R code*), removing one triad for which the proportion of eggs sired differed significantly between replicates (n = 20). So that sperm velocity in our model reflected conditions during the fertilisation trials, all VAP measures used were those activated in ovarian fluid, as female effects on sperm velocity can influence the outcome of sperm competition in chinook salmon (***Rosengrave et al., 2009b***, ***Rosengrave et al., 2016***).

All mentioned models used the week during the spawning season when milt samples were collected as a random predictor to control for potential seasonal effects on milt quality (***Butts et al., 2010***; ***Hajirezaee et al., 2010***), and the year fish were collected as a covariate (***Bolker, 2015***). To determine the significance of fixed effects, we present both 95% confidence intervals (CI) calculated using the Wald method, and p values calculated for linear mixed effects models with the package 'lmerTest' (***Kuznetsova et al., 2016***; RRID:SCR_015656) using Satterthwaite approximations to calculate degrees of freedom. Assumptions underlying parametric models were verified using residual plots and Shapiro tests. An alpha value of 0.05 was used to evaluate the significance of P-values and adjusted for multiple tests using the Bonferroni method. Refer to supplementary file: *Statistical analysis and R code*, for all R code used and output from analyses.

Statistical analysis and R code

Contains all R (***R Core Team, 2016***; RRID:SCR_001905) code, including output and model diagnostics. The following packages were used: lme4 (***Bates et al., 2015***; RRID:SCR_015654), lmerTest (***Kuznetsova et al., 2016***; RRID:SCR_015656), nlme (***Pinheiro et al., 2015***; RRID:SCR_015655), ggplot2 (***Wickham, 2009***; RRID:SCR_014601), lattice (***Sarkar, 2008***; RRID:SCR_015662), RVAideMemoire (***Hervé, 2016***; RRID:SCR_015657), LMERConvenienceFunctions (***Tremblay and Ransijn, 2015***; RRID:SCR_015658), and Deducer (***Fellows, 2012***; RRID:SCR_015659).

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Michael J Bartlett, Conceptualization, Data curation, Formal analysis, Validation, Investigation, Visualization, Writing—original draft, Writing—review and editing; Tammy E Steeves, Supervision, Project administration, Writing—review and editing; Neil J Gemmell, Conceptualization, Writing—review and editing; Patrice C Rosengrave, Conceptualization, Supervision, Funding acquisition, Investigation, Methodology, Project administration, Writing—review and editing

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Animal experimentation: All animals were collected and maintained according to the approved standards of the Animal Ethics Committee for the University of Otago, New Zealand.

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The following dataset was generated:

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Bartlett MJ, Steeves TE, Gemmell NJ, Rosengrave PC	2017	Data from: Sperm competition risk drives rapid ejaculate adjustments mediated by seminal fluiddx.doi.org/10.5061/dryad.kr137	Available at Dryad Digital Repository under a CC0 Public Domain Dedication

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APPENDIX B:

CHAPTER TWO: STATISTICAL ANALYSIS AND R CODE

STATISTICAL ANALYSIS AND R CODE

CONTENTS PAGE

R[1] (RRID:SCR_001905) **Packages used:**

```
library(ggplot2)[2]  #graphing (RRID:SCR_014601)

library(lattice)[3]  #graphing (RRID:SCR_015662)

library(lme4)[4]  #Linear mixed effects models (RRID:SCR_015654)

library(nlme)[5]  #Linear mixed effects models with specified variance
structures (RRID:SCR_015655)

library(lmerTest)[6]  #P value approximations for lmer (RRID:SCR_015656)

library(RVAideMemoire)[7]  #check for overdispersion in glmer (RRID:SCR_015657)

library(LMERConvenienceFunctions)[8]  #partial regression plots for mixed
effects models (RRID:SCR_015658)

library(Deducer)[9]  #Fishers exact and G tests (RRID:SCR_015659)
```

Models comparing in ejaculate parameters (VAP or sperm count) in males of Dominant (D) and Subdominant (S) social status from stage 1 of social status manipulation:

Linear mixed effects model with sperm velocity (VAP) as the response variable, social status and year as fixed predictors and male identity and week as random effects. Year was used as a fixed effect because it has fewer than 5 levels and is therefore unreliable as a random predictor[10].

```
VAPDIFFSSA<-
read.table(file="DIFF_VAP_SS_STAGE1.csv",header=T,row.names=NULL,sep=",")#
load Data sheet


model1<-lmer(VAP ~ SOCIAL.STATUS + as.factor(YEAR) + (1|WEEK)+
(1|MALE.ID), data=VAPDIFFSSA)
summary(model1)
```

```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula:
## VAP ~ SOCIAL.STATUS + as.factor(YEAR) + (1 | WEEK) + (1 | MALE.ID)
##    Data: VAPDIFFSSA
##
## REML criterion at convergence: 699.3
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.8588 -0.4541  0.0549  0.4637  1.9119
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MALE.ID  (Intercept) 712.46   26.692
##  WEEK     (Intercept)   8.59    2.931
##  Residual              40.90    6.395
## Number of obs: 87, groups:  MALE.ID, 44; WEEK, 5
##
## Fixed effects:
##                            Estimate Std. Error      df t value
Pr(>|t|)
## (Intercept)                152.860      8.970  21.400  17.041 6.24e-14
***
##
SOCIAL.STATUSsubdominant    7.370      8.167  36.670   0.902    0.373
##
as.factor(YEAR)2014        11.824      9.942  39.960   1.189    0.241
## as.factor(YEAR)2015        -1.832     11.130  38.810  -
0.165    0.870
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) SOCIAL a.(YEAR)2014
```

```
## SOCIAL.STAT  -0.455
## a.(YEAR)2014 -0.697  0.001
## a.(YEAR)2015 -0.620  0.000  0.560
```

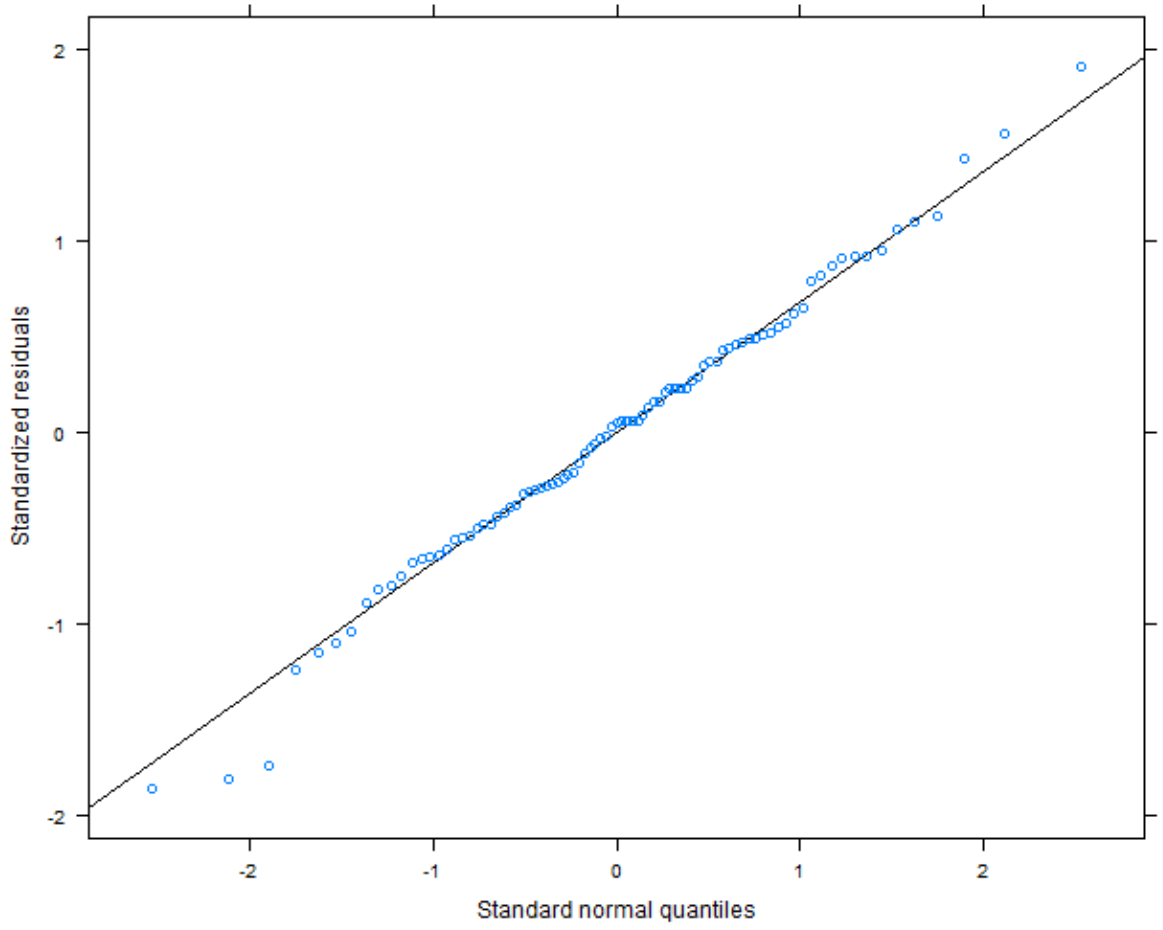
```
rand(model1)#Test for significance of random predictor male ID
```

```
## Analysis of Random effects Table:
##           Chi.sq Chi.DF p.value
## WEEK      0.0168      1     0.9
## MALE.ID 102.0088      1  <2e-16 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
confint(model1, level=0.95, method="Wald",oldNames=F) #generate 95%CI
using Wald method
```

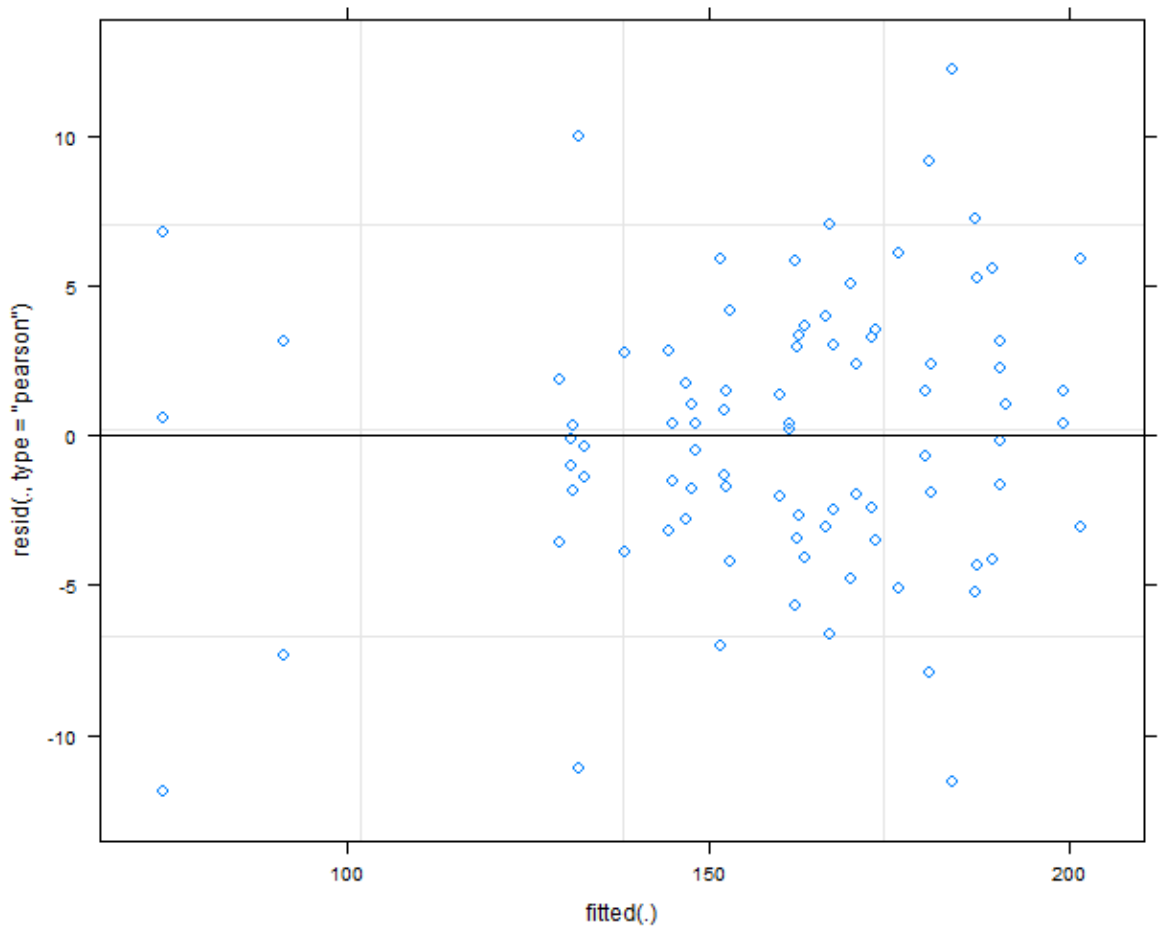
```
##                                 2.5 %    97.5 %
## sd_(Intercept)|MALE.ID             NA        NA
## sd_(Intercept)|WEEK                NA        NA
## sigma                              NA        NA
## (Intercept)                135.279207 170.44167
## SOCIAL.STATUSsubdominant    -8.636458  23.37664
## as.factor(YEAR)2014         -7.661824  31.31072
## as.factor(YEAR)2015        -23.646302  19.98171
```

```
qqmath(model1) #check normality assumption
```



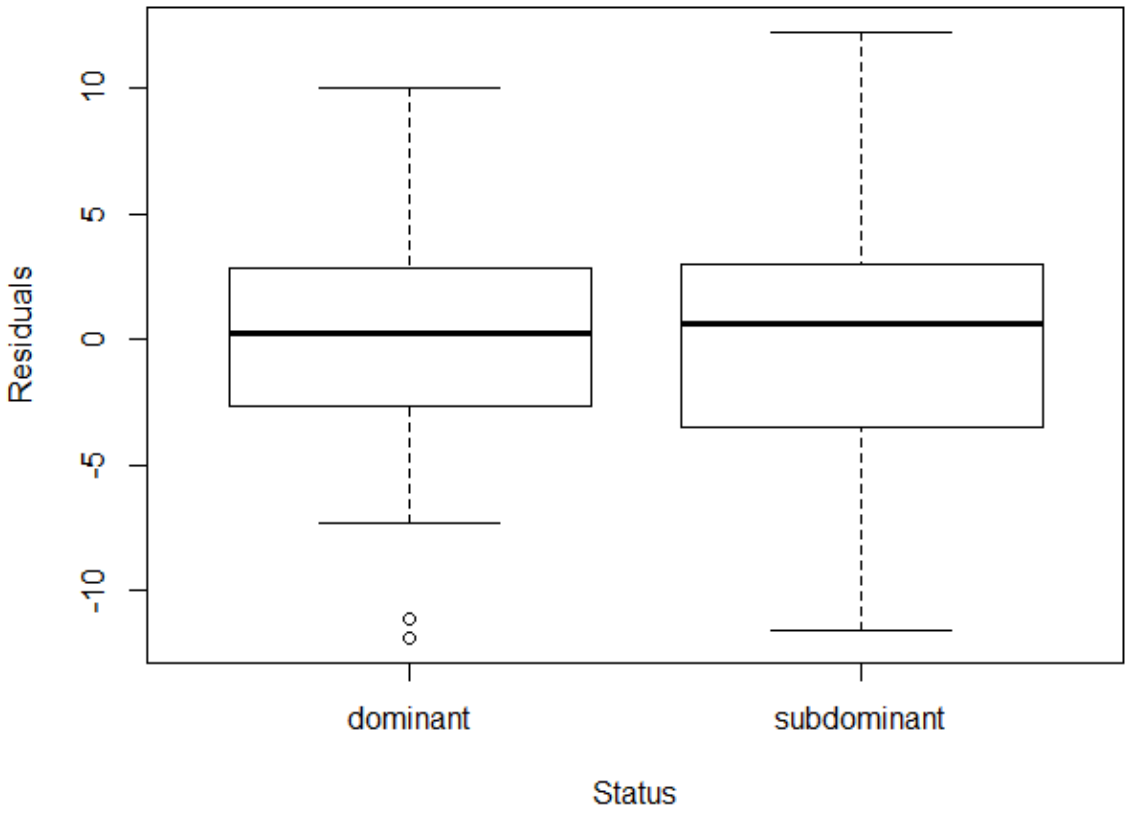

```
 shapiro.test(resid(model1))
##  Shapiro-Wilk normality test
## data:  resid(model1)
## W = 0.99043, p-value = 0.7805
```

```
plot(model1, results="hide", fig.show='hide') #plot residuals vs fitted values to check for unequal variance
```



Variance structure may be violating assumptions

```
plot(VAPDIFFSSA$SOCIAL.STATUS, resid(model1),
xlab="Status",ylab="Residuals")
```



Variance looks different for each social status group so will use lme() (nlme package) to implement different variances per level of status:

```
vf1<-varIdent(form= ~ 1|SOCIAL.STATUS)
M2<-lme(VAP ~ SOCIAL.STATUS + as.factor(YEAR), data =VAPDIFFSSA,random = ~
1|MALE.ID/WEEK, weights = vf1)
summary(M2)
```

```
## Linear mixed-effects model fit by REML
##  Data: VAPDIFFSSA
##        AIC      BIC    logLik
##   714.8842 734.2349 -349.4421
##
## Random effects:
##  Formula: ~1 | MALE.ID
##         (Intercept)
## StdDev:    19.00036
##
##  Formula: ~1 | WEEK %in% MALE.ID
##         (Intercept) Residual
## StdDev:    19.00036 6.838932
```

```
##
## Variance function:
##  Structure: Different standard deviations per stratum
##  Formula: ~1 | SOCIAL.STATUS
##  Parameter estimates:
## subdominant    dominant
##   1.0000000   0.8711948
## Fixed effects: VAP ~ SOCIAL.STATUS + as.factor(YEAR)
##                               Value Std.Error DF   t-value p-value
## (Intercept)              152.79580  8.864948 43 17.235950  0.0000
## SOCIAL.STATUSsubdominant   7.35512  8.221338 40  0.894638  0.3763
## as.factor(YEAR)2014       11.87273  9.954018 40  1.192758  0.2400
## as.factor(YEAR)2015       -2.14624 11.123055 40 -0.192954  0.8480
##  Correlation:
##                           (Intr) SOCIAL a.(YEAR)2014
## SOCIAL.STATUSsubdominant -0.462
## as.factor(YEAR)2014      -0.701  0.001
## as.factor(YEAR)2015      -0.627  0.000  0.559
##
## Standardized Within-Group Residuals:
##         Min          Q1         Med          Q3         Max
## -1.96121718 -0.48028457  0.03512917  0.46172327  1.79543625
##
## Number of Observations: 87
## Number of Groups:
##           MALE.ID WEEK %in% MALE.ID
##                44                44
```

Estimates for fixed effects are the same as for lmer() (lme4) so will report stats from simpler model.

Generalised Linear mixed effects model (Poisson) with sperm count as the response variable, social status and year as fixed predictors week as a random effect.

```
CountA<-
read.table(file="DIFF_COUNT_SS_STAGE1.csv",header=T,row.names=NULL,sep=","
)#load data frame

mod1<-glmer(COUNTRAW ~ as.factor(YEAR)+ SOCIAL.STATUS  +
(1|WEEK),  data=CountA, family="poisson")

## Residual deviance: 1430.696 on 37 degrees of freedom (ratio: 38.667)

dispersion <- 1:length(CountA$YEAR) #dispersion parameter for
overdispersed model

mod1.a<-glmer(COUNTRAW ~ as.factor(YEAR)+ SOCIAL.STATUS  + (1|WEEK) +
(1|dispersion),  data=CountA,family="poisson") #model was overdispersed so
added dispersion parameter to correct for overdispersion.
summary(mod1.a)

## Generalized linear mixed model fit by maximum likelihood (Laplace
##   Approximation) [glmerMod]
##  Family: poisson  ( log )
## Formula: COUNTRAW ~ as.factor(YEAR) + SOCIAL.STATUS + (1 | WEEK) +
##    (1| dispersion)
##
##    Data: CountA
##
##      AIC      BIC   logLik deviance df.resid
##    532.0    542.4   -260.0    520.0       36
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -0.45622 -0.15255  0.01699  0.10289  0.24218
##
## Random effects:
##  Groups     Name        Variance  Std.Dev.
##  dispersion (Intercept) 9.534e-02 3.088e-01
##  WEEK       (Intercept) 1.036e-10 1.018e-05
## Number of obs: 42, groups:  dispersion, 42; WEEK, 5
##
## Fixed effects:
##                            Estimate Std. Error z value Pr(>|z|)
## (Intercept)                 6.01889    0.10258   58.68  < 2e-16 ***
## as.factor(YEAR)2014        -0.43638    0.11691   -3.73 0.000189 ***
## as.factor(YEAR)2015         0.01945    0.12757    0.15 0.878805
## SOCIAL.STATUSSubdominant    0.19858    0.09694    2.05 0.040519 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##              (Intr) a.(YEAR)2014 a.(YEAR)2015
## a.(YEAR)2014 -0.701
```

```
## a.(YEAR)2015 -0.623  0.546
## SOCIAL.STAT  -0.476  0.047        0.002
```

```
overdisp.glmer(mod1.a)# Now underdispersed
```

```
## Residual deviance: 1.297 on 36 degrees of freedom (ratio: 0.036)
```

```
confint(mod1.a, level=0.95, method="Wald",oldNames=F) #generate 95%CI
using Wald method
```

```
##                                    2.5 %     97.5 %
## sd_(Intercept)|dispersion             NA         NA
## sd_(Intercept)|WEEK                   NA         NA
## (Intercept)                 5.817846528  6.2199398
## as.factor(YEAR)2014        -0.665519196 -0.2072460
## as.factor(YEAR)2015        -0.230583968  0.2694893
## SOCIAL.STATUSSubdominant    0.008575323  0.3885871
```

```
#Test significance of random effects
mod1.a2<-glmer(COUNTRAW ~ as.factor(YEAR)+ SOCIAL.STATUS +
(1|dispersion),  data=CountA,family="poisson")
anova(mod1.a,mod1.a2)
```

```
## Data: CountA
## Models:
## mod1.a2: COUNTRAW ~ as.factor(YEAR) + SOCIAL.STATUS + (1 | dispersion)
## mod1.a: COUNTRAW ~ as.factor(YEAR) + SOCIAL.STATUS + (1 | WEEK) + (1 |
## mod1.a:     dispersion)
##         Df AIC    BIC logLik deviance Chisq Chi Df Pr(>Chisq)
## mod1.a2  5 530 538.69   -260      520
## mod1.a   6 532 542.43   -260      520     0      1     0.9999
```

Models comparing ejaculate parameters (VAP or sperm count) in males of Dominant (D) and Subdominant (S) social status from stage 2 of social status manipulation:

Linear mixed effects model with sperm velocity (VAP) as the response variable, social status and year as fixed predictors and male identity and week as random effects.

```
VAPDIFFSSB<-read.table(file="
DIFF_VAP_SS_STAGE2.csv",header=T,row.names=NULL,sep=",")#Load data frame
```

Start with social status as factor with 2 levels, S (SS and DS pooled) and D (DD and SD pooled).

```
model2<-lmer(VAP ~ as.factor(YEAR) + SOCIAL.STATUS + (1|WEEK)+
(1|MALE.ID), data=VAPDIFFSSB) #D and S males pooled
summary(model2)
```

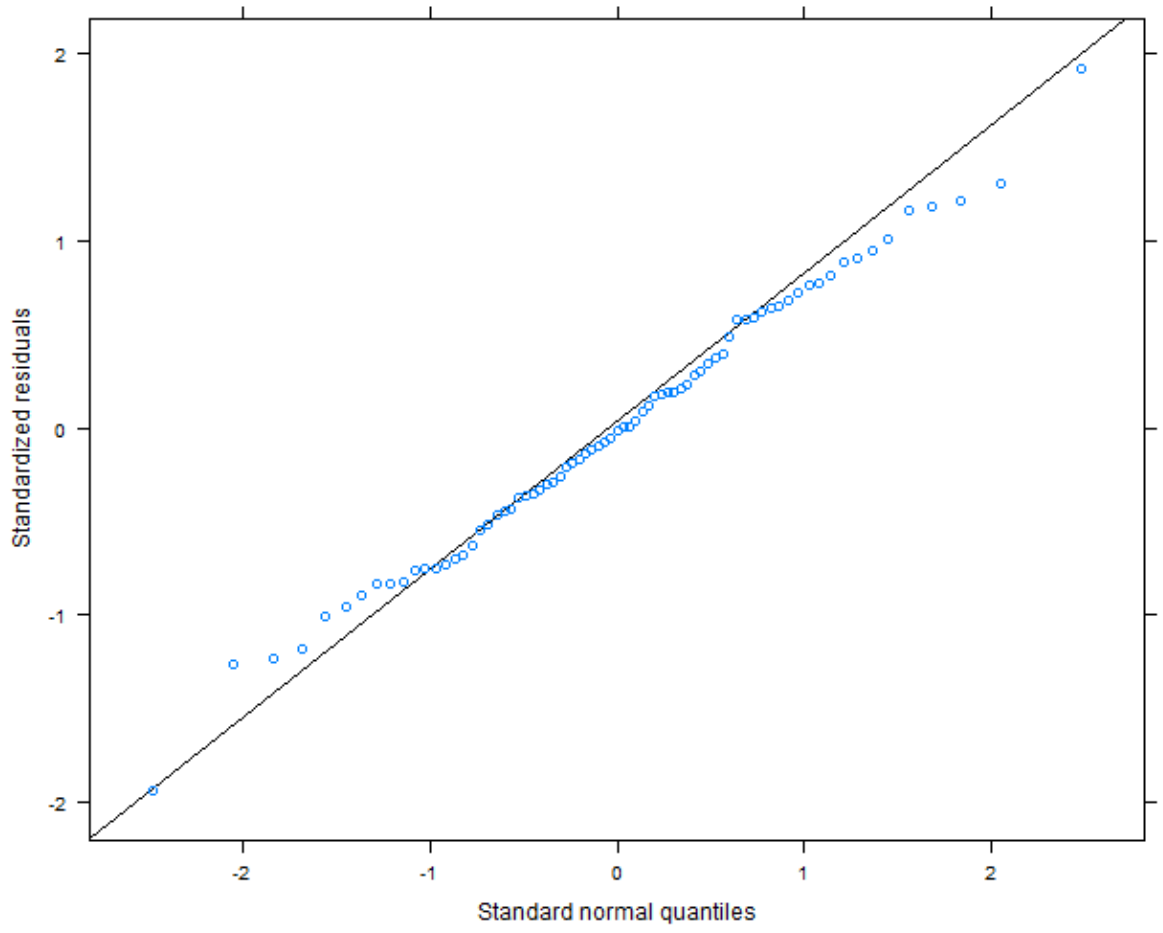
```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula:
## VAP ~ as.factor(YEAR) + SOCIAL.STATUS + (1 | WEEK) + (1 | MALE.ID)
##    Data: VAPDIFFSSB
##
## REML criterion at convergence: 581.3
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.93909 -0.48952 -0.00969  0.58019  1.92393
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MALE.ID  (Intercept) 503.62   22.441
##  WEEK     (Intercept)  75.83    8.708
##  Residual              32.73    5.721
## Number of obs: 75, groups:  MALE.ID, 38; WEEK, 5
##
## Fixed effects:
##                          Estimate Std. Error      df t value Pr(>|t|)
## (Intercept)               127.147      9.373  18.030  13.566 6.68e-11 ***
## as.factor(YEAR)2014        34.192      9.633  34.010   3.550  0.00115 **
## as.factor(YEAR)2015        29.836     10.421  33.640   2.863  0.00717 **
## SOCIAL.STATUSsubdominant   19.653      7.401  30.870   2.655  0.01242 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##              (Intr) a.(YEAR)2014 a.(YEAR)2015
## a.(YEAR)2014 -0.656
## a.(YEAR)2015 -0.606  0.593
## SOCIAL.STAT  -0.395  0.001        0.000
```

```
confint(model2, level=0.95, method="Wald",oldNames=F) #generate 95%CI
using Wald method
```

```
##                                  2.5 %    97.5 %
## sd_(Intercept)|MALE.ID              NA        NA
## sd_(Intercept)|WEEK                 NA        NA
## sigma                               NA        NA
## (Intercept)                108.776923 145.51751
## as.factor(YEAR)2014         15.312380  53.07158
## as.factor(YEAR)2015          9.410564  50.26056
## SOCIAL.STATUSsubdominant     5.147170  34.15966
```

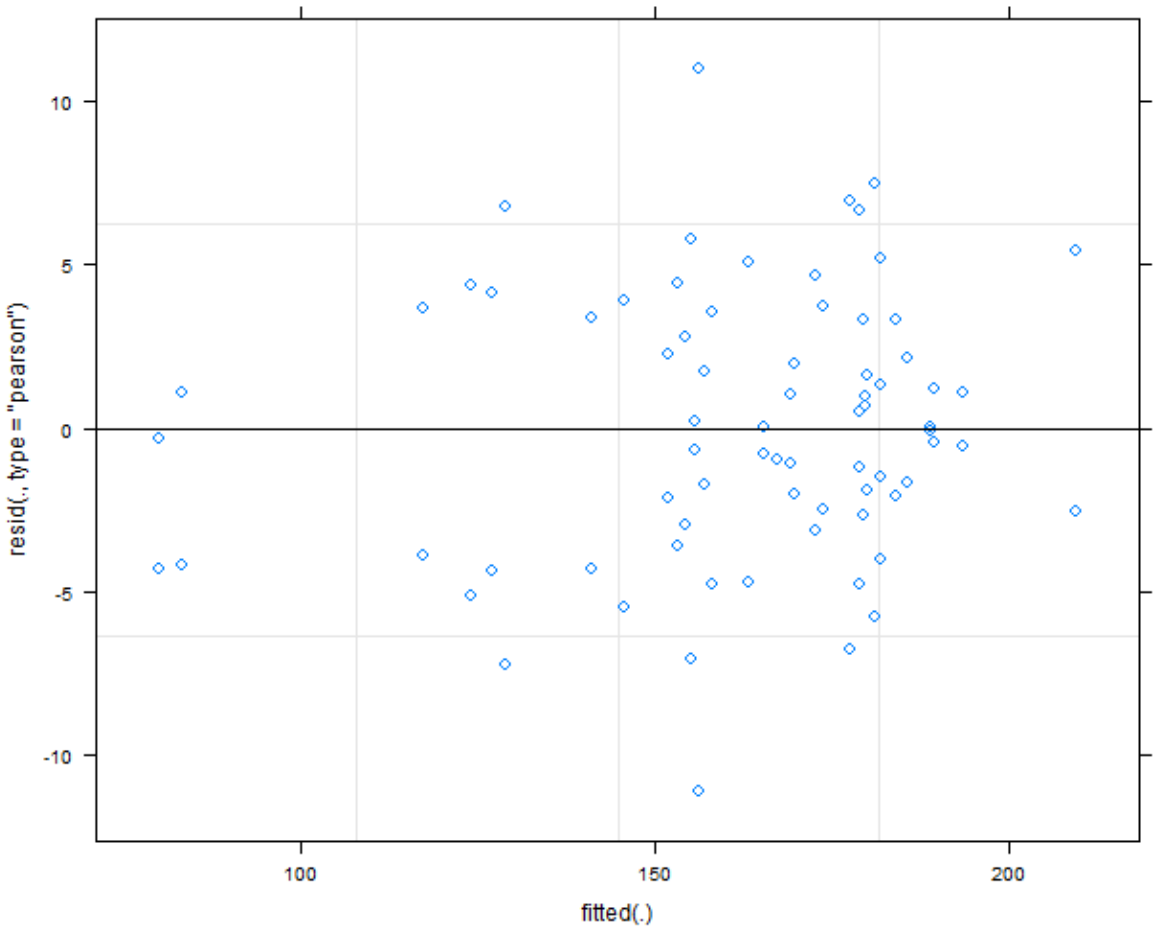
```
qqmath(model2) #check normality assumption
```

```
shapiro.test(resid(model2))
```




```
##  Shapiro-Wilk normality test
## data:  resid(model2)
## W = 0.99423, p-value = 0.9838
```

```
plot(model2, results="hide", fig.show='hide') #plot residuals vs fitted values to check for unequal variance
```




```
#Test significance of random effects
rand(model2)
```

```
## Analysis of Random effects Table:
##         Chi.sq Chi.DF p.value    
## WEEK      1.49      1     0.2    
## MALE.ID  76.91      1  <2e-16 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Now use factor SSGROUP with four levels (DD,DS,SD,SS).

This model was run 3 times using the relevel code below*** to change the social status group that each other group is compared to, first using DD, then DS and then SD. The results are collated below.

e.g.***VAPDIFFSSB$SS.GROUP<-**relevel**(VAPDIFFSSB$SS.GROUP,"DD")*** # this model compares group DD to each other group.

```
model2.1<-lmer(VAP ~ as.factor(YEAR) + SS.GROUP + (1|WEEK)+ (1|MALE.ID), data=VAPDIFFSSB) #four social status groups DD, SD, DS, SS
summary(model2)
```

```
Linear mixed model fit by REML t-tests use Satterthwaite approximations to degrees of freedom [lmerMod]
Formula: VAP ~ as.factor(YEAR) + SS.GROUP + (1 | WEEK) + (1 | MALE.ID)
  Data: VAPDIFFSSB

REML criterion at convergence: 567.7

Scaled residuals:
    Min      1Q  Median      3Q     Max
-1.92015 -0.50118  0.00319  0.59748  1.94278

Random effects:
Groups   Name        Variance Std.Dev.
MALE.ID  (Intercept) 535.73   23.146
WEEK     (Intercept)  66.02    8.125
Residual              32.73    5.721
Number of obs: 75, groups:  MALE.ID, 38; WEEK, 5

Fixed effects:
                   Estimate Std. Error      df t value Pr(>|t|)
(Intercept)          131.232     11.227  24.030  11.689 2.11e-11 ***
as.factor(YEAR)2014   32.621     10.076  31.970   3.238  0.00281 **
as.factor(YEAR)2015   29.003     10.708  31.530   2.708  0.01083 *
SS.GROUP DD - DS      17.895     10.516  29.070   1.702  0.09947 .
SS.GROUP DD - SD      -6.513     10.969  29.780  -0.594  0.55717
SS.GROUP DD - SS      14.982     10.969  29.780   1.366  0.18222
SS.GROUP DS - SD     -24.408     10.974  29.830  -2.224  0.03386 *
SS.GROUP DS - SS      -2.914     10.974  29.830  -0.265  0.79246
SS.GROUP SD - SS      21.494     11.076  28.990   1.941  0.06209 .

---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

No significant results after applying p value correction (alpha = 0.016)

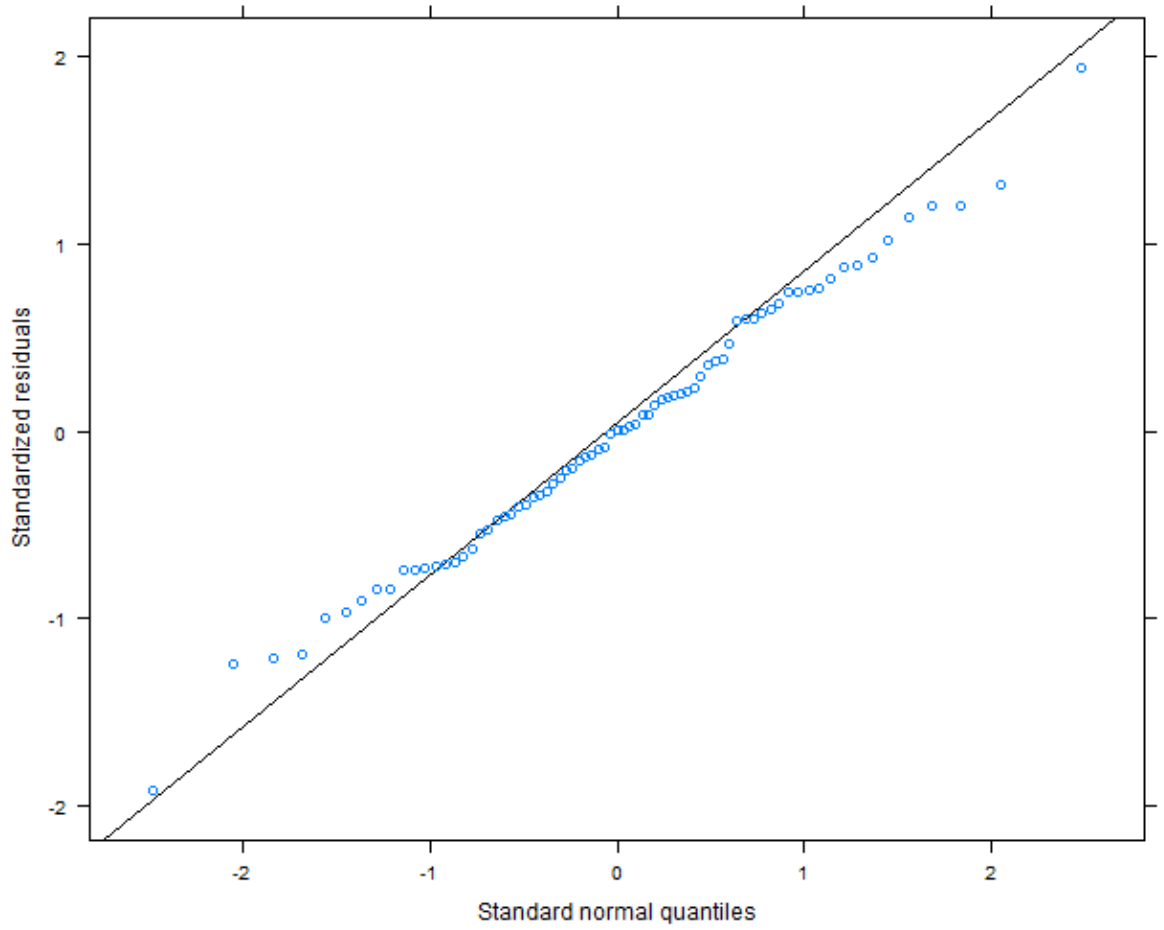
```
Correlation of Fixed Effects:
            (Intr) a.(YEAR)2014 a.(YEAR)2015 SS.GROUP D SS.GROUP SD
a.(YEAR)2014 -0.634
a.(YEAR)2015 -0.555  0.596
SS.GROUP DS  -0.468  0.001        0.000
```

```
SS.GROUP SD  -0.534  0.144        0.067        0.479
SS.GROUP SS  -0.534  0.144        0.067        0.479      0.490
```

```
confint(model2.1, level=0.95, method="Wald",oldNames=F) #generate 95%CI
using Wald method
```

```
                            2.5 %    97.5 %
sd_(Intercept)|MALE.ID         NA        NA
sd_(Intercept)|WEEK            NA        NA
sigma                          NA        NA
(Intercept)            109.227477 153.23556
as.factor(YEAR)2014     12.873665  52.36927
as.factor(YEAR)2015      8.014878  49.99089
SS.GROUP DD – DS        -2.715183  38.50554
SS.GROUP DD - SD       -28.011627  14.98605
SS.GROUP DD - SS        -6.517183  36.48049
SS.GROUP DS - SD       -45.916823  -2.899110
SS.GROUP DS - SS       -24.422378  18.595334
SS.GROUP SD - SS        -0.215076  43.20396
```

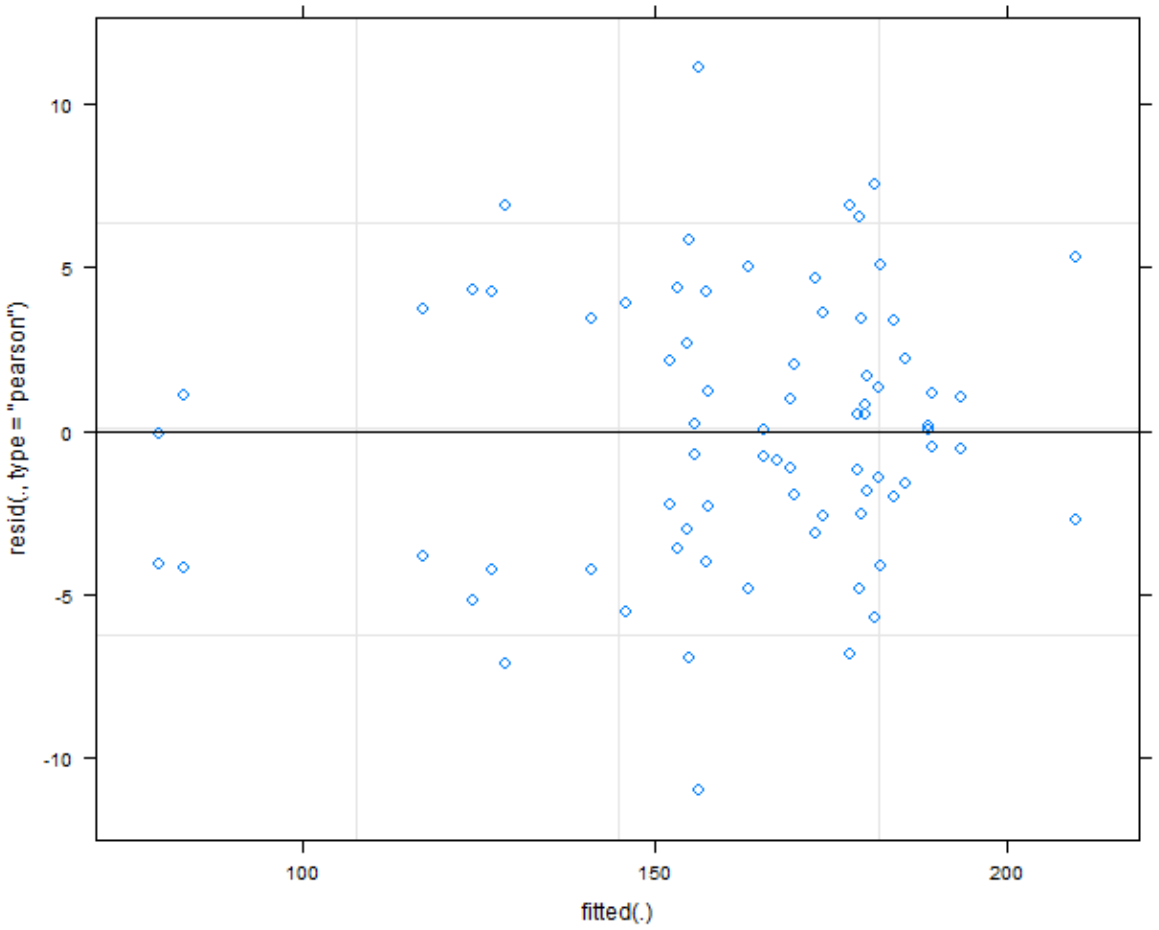
```
qqmath(model2) #check normality assumption
```



```
shapiro.test(resid(model2.1))
```

```
	Shapiro-Wilk normality test

data:  resid(model2)
W = 0.9931, p-value = 0.961
```

```
plot(model2.1, results="hide", fig.show='hide') #plot residuals vs fitted values to check for unequal variance
```



Generalised linear mixed effects model with sperm count as the response variable, social status and year as fixed predictors and week as a random effect. A poisson error distribution is used for count data

```
CountB<-read.table(file="
DIFF_COUNT_SS_STAGE2.csv",header=T,row.names=NULL,sep=",")#load data frame
```

Start with social status as factor with 2 levels, S (SS and DS pooled) and D (DD and SD pooled).

```
mod2<-glmer(COUNTRAW ~  as.factor(YEAR) + SOCIAL.STATUS  +
(1|WEEK),  data=CountB, family="poisson")


overdisp.glmer(mod2)# check for over/under-dispersion
```

```
## Residual deviance: 2087.387 on 34 degrees of freedom (ratio: 61.394)
```

```
dispersion2<-1:length(CountB$YEAR)# disperion parameter for overdispersed
model

mod2.a<-glmer(COUNTRAW ~  as.factor(YEAR) + SOCIAL.STATUS  + (1|WEEK) +
(1|dispersion2),  data=CountB, family="poisson") #model overdispersed so
added dispersion parameter
summary(mod2.a)
```

```
## Generalized linear mixed model fit by maximum likelihood (Laplace
##   Approximation) [glmerMod]
##  Family: poisson  ( log )
## Formula: COUNTRAW ~ as.factor(YEAR) + SOCIAL.STATUS + (1 | WEEK) +
##  (1 | dispersion2)
##    Data: CountB
##
##      AIC      BIC   logLik deviance df.resid
##    501.4    511.4   -244.7    489.4       33
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -0.30444 -0.13054 -0.01825  0.09442  0.20362
##
## Random effects:
##  Groups      Name        Variance Std.Dev.
##  dispersion2 (Intercept) 0.1365   0.3694
##  WEEK        (Intercept) 0.0000   0.0000
## Number of obs: 39, groups:  dispersion2, 39; WEEK, 5
##
## Fixed effects:
##                           Estimate Std. Error z value Pr(>|z|)
## (Intercept)                 5.9688     0.1250   47.77   <2e-16 ***
## as.factor(YEAR)2014        -0.3301     0.1463   -2.26   0.0241 *
## as.factor(YEAR)2015        -0.2475     0.1559   -1.59   0.1125
## SOCIAL.STATUSSubdominant    0.1754     0.1198    1.46   0.1431
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
```

```
## Correlation of Fixed Effects:
##              (Intr) a.(YEAR)2014 a.(YEAR)2015
## a.(YEAR)2014 -0.675
## a.(YEAR)2015 -0.633  0.555
## SOCIAL.STAT  -0.436 -0.037       -0.036
```

```
overdisp.glmer(mod2.a)# check for over/under-dispersion
```

```
## Residual deviance: 0.77 on 33 degrees of freedom (ratio: 0.023)
```

```
confint(mod2.a, level=0.95, method="Wald",oldNames=F) #generate 95%CI
using Wald method
```

```
##                                    2.5 %      97.5 %
## sd_(Intercept)|dispersion2            NA          NA
## sd_(Intercept)|WEEK                   NA          NA
## (Intercept)                 5.72387849  6.21371400
## as.factor(YEAR)2014        -0.61685909 -0.04326561
## as.factor(YEAR)2015        -0.55304392  0.05810625
## SOCIAL.STATUSSubdominant   -0.05937055  0.41014065
```

Now use factor SSGROUP with four levels (DD,DS,SD,SS).

This model was run 3 times using the relevel code below*** to change the social status group that each other group is compared to, first using DD, then DS and then SD. The results are collated below.

```
***CountB$SSGROUP <-relevel(CountB$SSGROUP,"DD")***
```

```
mod2.b<-glmer(COUNTRAW ~  as.factor(YEAR) + SSGROUP  + (1|WEEK) +
(1|dispersion2),  data=CountB, family="poisson") #model overdispersed so
added dispersion parameter
summary(mod2.b)
```

```
## Generalized linear mixed model fit by maximum likelihood (Laplace
##   Approximation) [glmerMod]
##  Family: poisson  ( log )
## Formula:
## COUNTRAW ~ as.factor(YEAR) + SSGROUP + (1 | WEEK) + (1 | dispersion2)
##    Data: CountB
##
##      AIC      BIC   logLik deviance df.resid
##    498.9    512.2   -241.5    482.9       31
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -0.34875 -0.15248 -0.00253  0.08914  0.24087
##
## Random effects:
##  Groups      Name        Variance Std.Dev.
##  dispersion2 (Intercept) 0.1151   0.3392
##  WEEK        (Intercept) 0.0000   0.0000
## Number of obs: 39, groups:  dispersion2, 39; WEEK, 5
##
```

```
## Fixed effects:
##                     Estimate Std. Error z value Pr(>|z|)
## (Intercept)           5.8232     0.1373   42.42  < 2e-16 ***
## as.factor(YEAR)2014  -0.2773     0.1361   -2.04  0.04159 *
## as.factor(YEAR)2015  -0.2318     0.1436   -1.61  0.10651
## SSGROUP DD - DS       0.1388     0.1507    0.92  0.35709
## SSGROUP DD - SD       0.2665     0.1552    1.72  0.08606 .
## SSGROUP DD - SS       0.4659     0.1550    3.01  0.00265 **
## SSGROUP DS - SD       0.1277     0.1593    0.80  0.4227
## SSGROUP DS - SS       0.3271     0.1590    2.06  0.0397  *
## SSGROUP SD - SS      -0.1994     0.1617   -1.23  0.21745
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

DD – SS significant result after applying p value correction (alpha = 0.016)

```
## Correlation of Fixed Effects:
##              (Intr) a.(YEAR)2014 a.(YEAR)2015 SSGROUPDS SSGROUPSD
## a.(YEAR)2014 -0.610
## a.(YEAR)2015 -0.535  0.555
## SSGROUP DS   -0.483 -0.054       -0.051
## SSGROUP SD   -0.543  0.078        0.004        0.458
## SSGROUP SS   -0.543  0.077        0.003        0.459       0.457
```

```
overdisp.glmer(mod2.b)# check for over/under-dispersion
```

```
## Residual deviance: 0.892 on 31 degrees of freedom (ratio: 0.029)
```

```
confint(mod2.b, level=0.95, method="Wald",oldNames=F) #generate 95%CI
using Wald method
```

```
##                                    2.5 %      97.5 %
## sd_(Intercept)|dispersion2            NA          NA
## sd_(Intercept)|WEEK                   NA          NA
## (Intercept)                 5.55411444  6.09225136
## as.factor(YEAR)2014        -0.54411128 -0.01056046
## as.factor(YEAR)2015        -0.51331918  0.04967555
## SSGROUP DD - DS            -0.15659105  0.43417286
## SSGROUP DD - SD            -0.03778601  0.57075869
## SSGROUP DD - SS             0.16210528  0.76975328
## SSGROUP DS - SD            -0.18448161  0.43988726
## SSGROUP DS - SS             0.01540979  0.63887362
## SSGROUP SD - SS            -0.5163898  0.11750514
```

Models to compare changes in ejaculate parameters (VAP or sperm count) in males from each social group from stage 1 to 2:

Model that compares mean VAP for males that are D (stage 1) that become DS (stage 2).

```
CHANGEDTODSVAP<-read.table(file="
CHANGE_VAP_D_TO_DD.csv",header=T,row.names=NULL,sep=",")#load data frame

model4<-lmer(VAP ~ as.factor(YEAR) + SOCIAL.STATUS + (1|WEEK) +
(1|MALE.ID) ,data=CHANGEDTODSVAP)
summary(model4)
```

```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula:
## VAP ~ as.factor(YEAR) + SOCIAL.STATUS + (1 | WEEK) + (1 | MALE.ID)
##    Data: CHANGEDTODSVAP
##
## REML criterion at convergence: 303
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -2.14236 -0.41628 -0.06181  0.69382  1.67821
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MALE.ID  (Intercept)   2.851   1.689
##  WEEK     (Intercept) 345.651  18.592
##  Residual             333.371  18.258
## Number of obs: 37, groups:  MALE.ID, 10; WEEK, 5
##
## Fixed effects:
##                     Estimate Std. Error      df t value Pr(>|t|)
## (Intercept)          163.920     11.667   6.878  14.050 2.56e-06 ***
## as.factor(YEAR)2014  -13.500      8.970   3.299  -1.505   0.2213
## as.factor(YEAR)2015  -10.565     10.099   3.283  -1.046   0.3663
## SOCIAL.STATUSDS       17.242      6.050  26.292   2.850   0.0084 **
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

**remains significant following P-value correction (alpha = 0.0125)

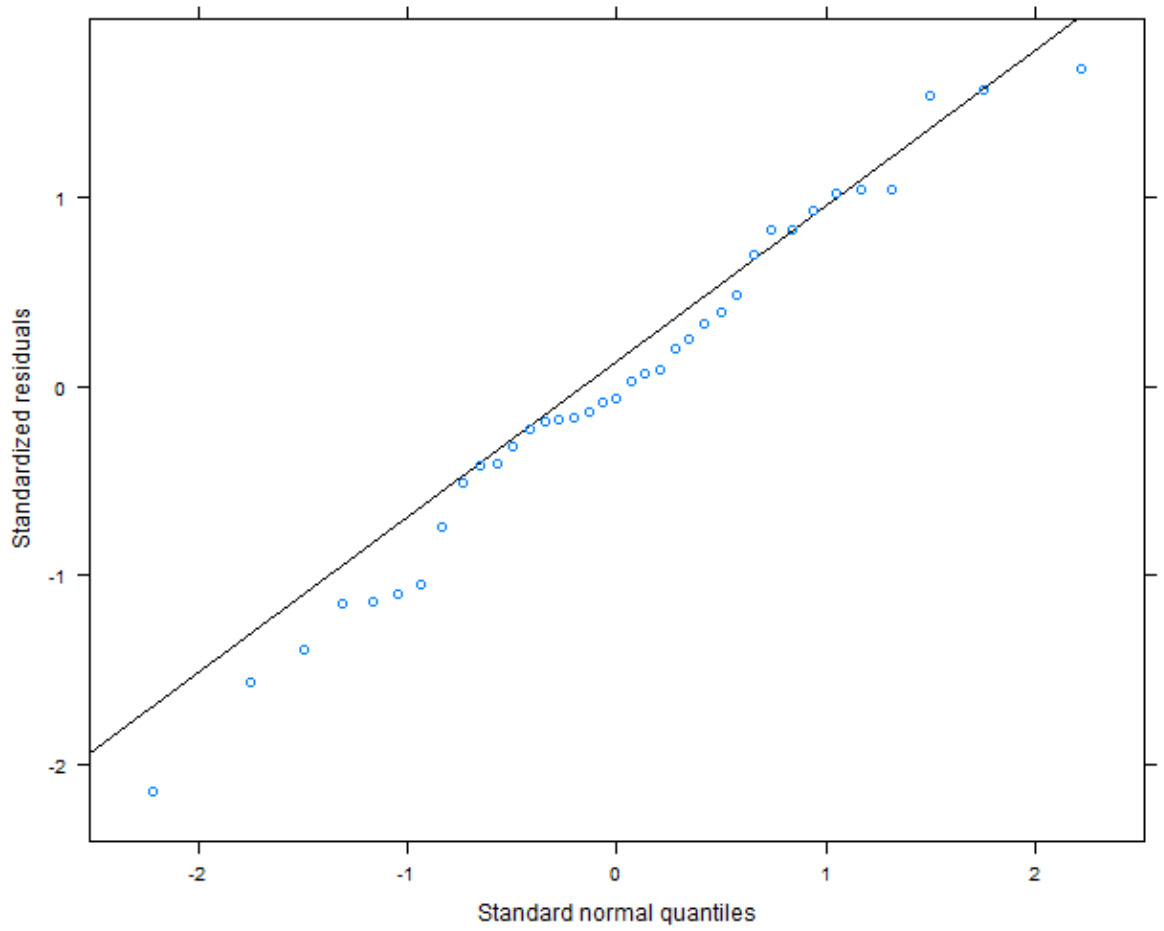
```
##
## Correlation of Fixed Effects:
##              (Intr) a.(YEAR)2014 a.(YEAR)2015
## a.(YEAR)2014 -0.569
## a.(YEAR)2015 -0.515  0.685
## SOCIAL.STAT  -0.228 -0.019       -0.062
```

```
confint(model4, level=0.95, method="Wald",oldNames=F) #generate 95%CI
using Wald method
```

```
                                2.5 %     97.5 %
## sd_(Intercept)|MALE.ID         NA         NA
```

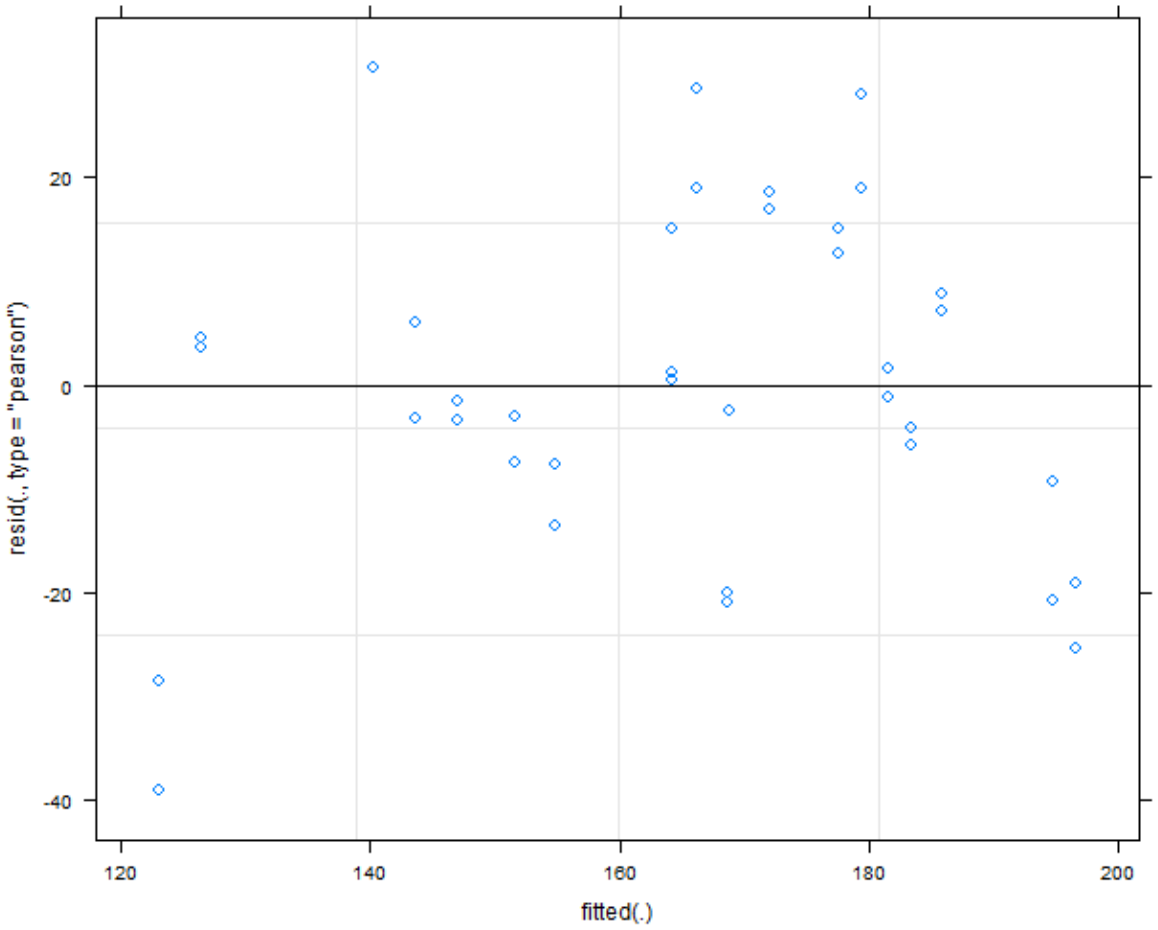
```
## sd_(Intercept)|WEEK             NA         NA
## sigma                           NA         NA
## (Intercept)             141.053996 186.786069
## as.factor(YEAR)2014     -31.081606   4.081081
## as.factor(YEAR)2015     -30.358576   9.228218
## SOCIAL.STATUSDS           5.383677  29.100929
```

```
qqmath(model4) #check normality assumption
```




```
shapiro.test(resid(model4))
##  Shapiro-Wilk normality test
## data:  resid(model4)
## W = 0.98145, p-value = 0.7808
```

```
plot(model4, results="hide", fig.show='hide') #plot residuals vs fitted
values to check for unequal variance
```




```
#Test for significance of random predictor male ID
model4.1<-lmer(VAP ~ as.factor(YEAR) + SOCIAL.STATUS + (1|WEEK)
,data=CHANGEDTODSVAP)
anova(model4,model4.1)

## refitting model(s) with ML (instead of REML)

## Data: CHANGEDTODSVAP
## Models:
## ..1: VAP ~ as.factor(YEAR) + SOCIAL.STATUS + (1 | WEEK)
## object: VAP ~ as.factor(YEAR) + SOCIAL.STATUS + (1 | WEEK) + (1 |
MALE.ID)
##        Df    AIC    BIC  logLik deviance Chisq Chi Df Pr(>Chisq)
## ..1     6 338.36 348.02 -163.18   326.36
## object  7 340.36 351.63 -163.18   326.36     0      1          1
```

Model that compares mean VAP for males that are D (stage 1) that become DD (stage 2).

```
CHANGEDTODDVAP<-read.table(file="
CHANGE_VAP_D_TO_DD.csv",header=T,row.names=NULL,sep=",")#load data frame

model5<-lmer(VAP ~ as.factor(YEAR) + SOCIAL.STATUS  + (1|WEEK) +
(1|MALE.ID) ,data=CHANGEDTODDVAP)
summary(model5)
```

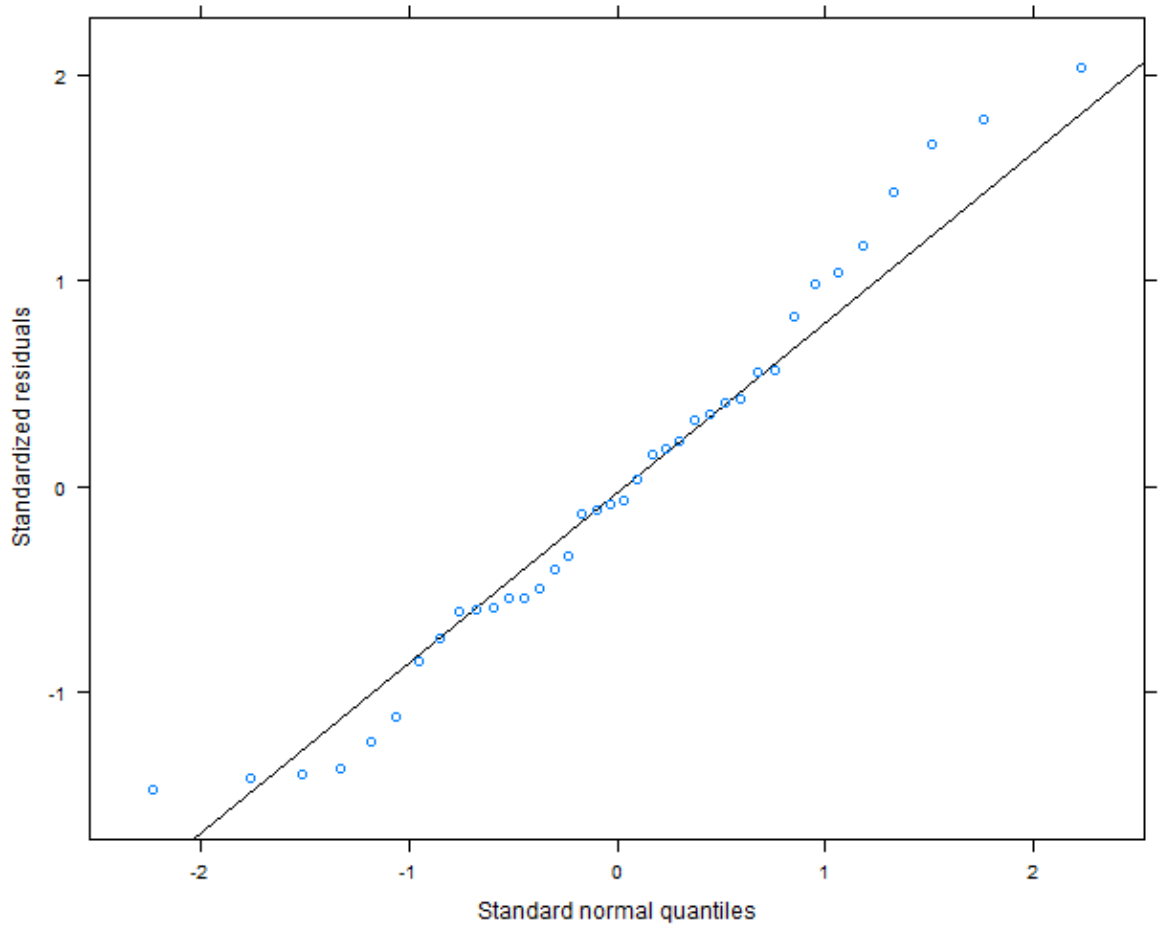
```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula:
## VAP ~ as.factor(YEAR) + SOCIAL.STATUS + (1 | WEEK) + (1 | MALE.ID)
##    Data: CHANGEDTODDVAP
##
## REML criterion at convergence: 320.1
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.4747 -0.5969 -0.0803  0.5212  2.0354
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MALE.ID  (Intercept)  60.49    7.777
##  WEEK     (Intercept)   0.00    0.000
##  Residual             499.21   22.343
## Number of obs: 38, groups:  MALE.ID, 10; WEEK, 5
##
## Fixed effects:
##                     Estimate Std. Error       df t value Pr(>|t|)
## (Intercept)         109.0623    10.2910   8.2350  10.598 4.41e-06 ***
## as.factor(YEAR)2014  60.0995    11.5247   6.5910   5.215  0.00149 **
## as.factor(YEAR)2015  60.4750    12.4261   6.3210   4.867  0.00243 **
## SOCIAL.STATUSDD       0.1255     7.2826  27.3450   0.017  0.98638
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) a.(YEAR)2014 a.(YEAR)2015
## a.(YEAR)2014 -0.768
## a.(YEAR)2015 -0.724  0.647
## SOCIAL.STAT  -0.354 -0.037        0.000
```

```
confint(model5, level=0.95, method="Wald",oldNames=F) #generate 95%CI
using Wald method
```

```
##                             2.5 %    97.5 %
## sd_(Intercept)|MALE.ID         NA        NA
## sd_(Intercept)|WEEK            NA        NA
## sigma                          NA        NA
## (Intercept)             88.89237 129.23217
## as.factor(YEAR)2014     37.51157  82.68750
```

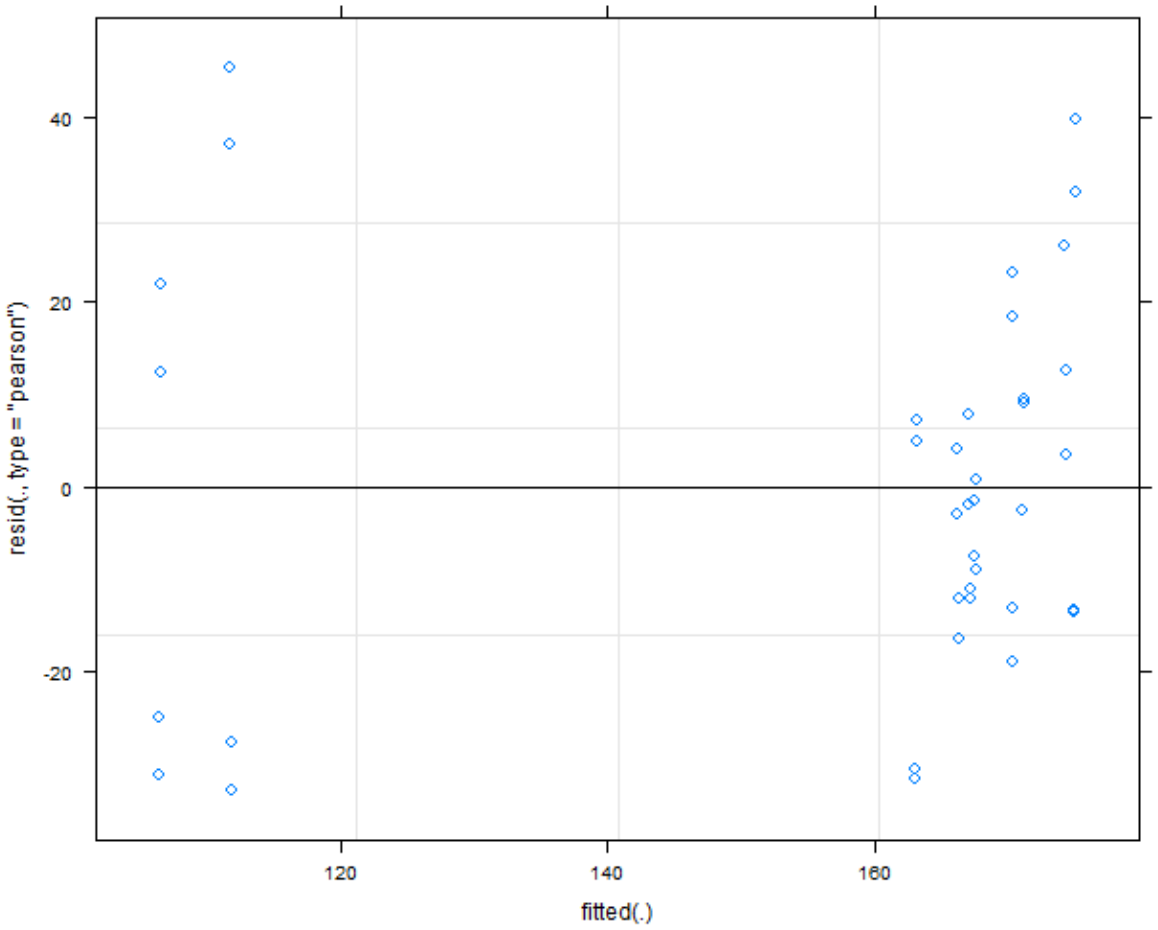
```
## as.factor(YEAR)2015     36.12032  84.82968
## SOCIAL.STATUSDD        -14.14826  14.39919
```

```
qqmath(model5) #check normality assumption
```




```
shapiro.test(resid(model5))
##  Shapiro-Wilk normality test
## data:  resid(model5)
## W = 0.96978, p-value = 0.3857
```

```
plot(model5, results="hide", fig.show='hide') #plot residuals vs fitted
values to check for unequal variance
```




```
#Test for significance of random predictor male ID
model5.1<-lmer(VAP ~ as.factor(YEAR) + SOCIAL.STATUS  + (1|WEEK)
,data=CHANGEDTODDVAP)
anova(model5,model5.1)

## refitting model(s) with ML (instead of REML)

## Data: CHANGEDTODDVAP
## Models:
## ..1: VAP ~ as.factor(YEAR) + SOCIAL.STATUS + (1 | WEEK)
## object: VAP ~ as.factor(YEAR) + SOCIAL.STATUS + (1 | WEEK) + (1 |
MALE.ID)
##        Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
## ..1     6 355.10 364.92 -171.55   343.10
## object  7 357.09 368.56 -171.55   343.09 0.0043      1     0.9476
```

Model that compares mean VAP for males that are S (stage 1) that become SD (stage 2).

```
CHANGESTOSDVAP<-read.table(file="
CHANGE_VAP_S_TO_SD.csv",header=T,row.names=NULL,sep=",")#Load data frame
model6<-lmer(VAP ~  as.factor(YEAR) +SOCIAL.STATUS+ (1|WEEK) + (1|MALE.ID)
,data=CHANGESTOSDVAP)
summary(model6)
```

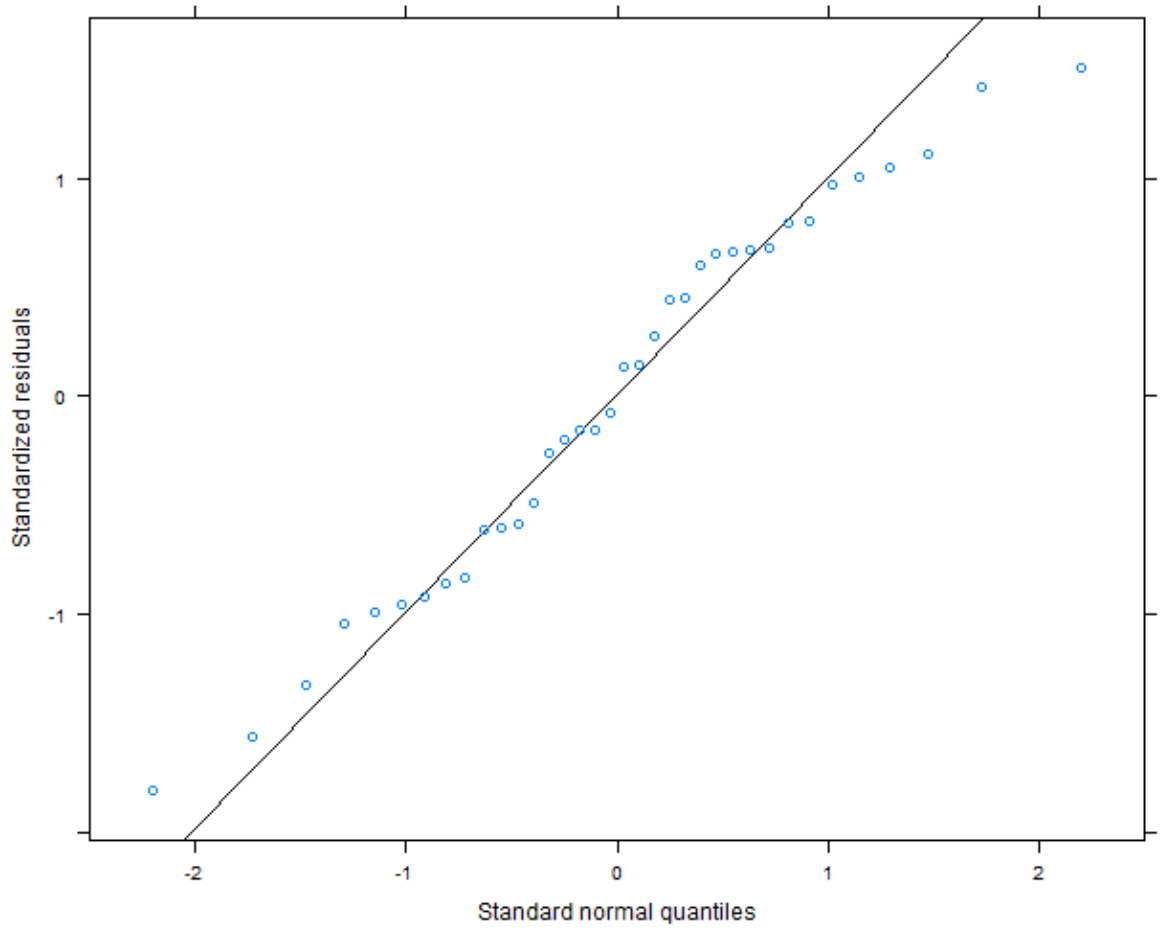
```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula:
## VAP ~ as.factor(YEAR) + SOCIAL.STATUS + (1 | WEEK) + (1 | MALE.ID)
##    Data: CHANGESTOSDVAP
##
## REML criterion at convergence: 291.6
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.80659 -0.66496  0.02879  0.67560  1.50712
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MALE.ID  (Intercept) 511.3    22.61
##  WEEK     (Intercept)   0.0     0.00
##  Residual             261.0    16.16
## Number of obs: 36, groups:  MALE.ID, 9; WEEK, 5
##
## Fixed effects:
##                     Estimate Std. Error      df t value Pr(>|t|)
## (Intercept)          139.553     14.122   6.459   9.882  3.9e-05 ***
## as.factor(YEAR)2014   30.300     19.605   6.000   1.546    0.173
## as.factor(YEAR)2015   14.742     19.605   6.000   0.752    0.481
## SOCIAL.STATUSSD       -8.972      5.385  26.000  -1.666    0.108
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) a.(YEAR)2014 a.(YEAR)2015
## a.(YEAR)2014 -0.694
## a.(YEAR)2015 -0.694  0.500
## SOCIAL.STAT  -0.191  0.000        0.000
```

```
confint(model6, level=0.95, method="Wald",oldNames=F) #generate 95%CI
using Wald method
```

```
##                               2.5 %     97.5 %
## sd_(Intercept)|MALE.ID           NA         NA
## sd_(Intercept)|WEEK              NA         NA
## sigma                            NA         NA
## (Intercept)             111.874737 167.230819
## as.factor(YEAR)2014      -8.124501  68.724501
```

```
## as.factor(YEAR)2015    -23.682835  53.166168
## SOCIAL.STATUSSD        -19.527376   1.582931
```

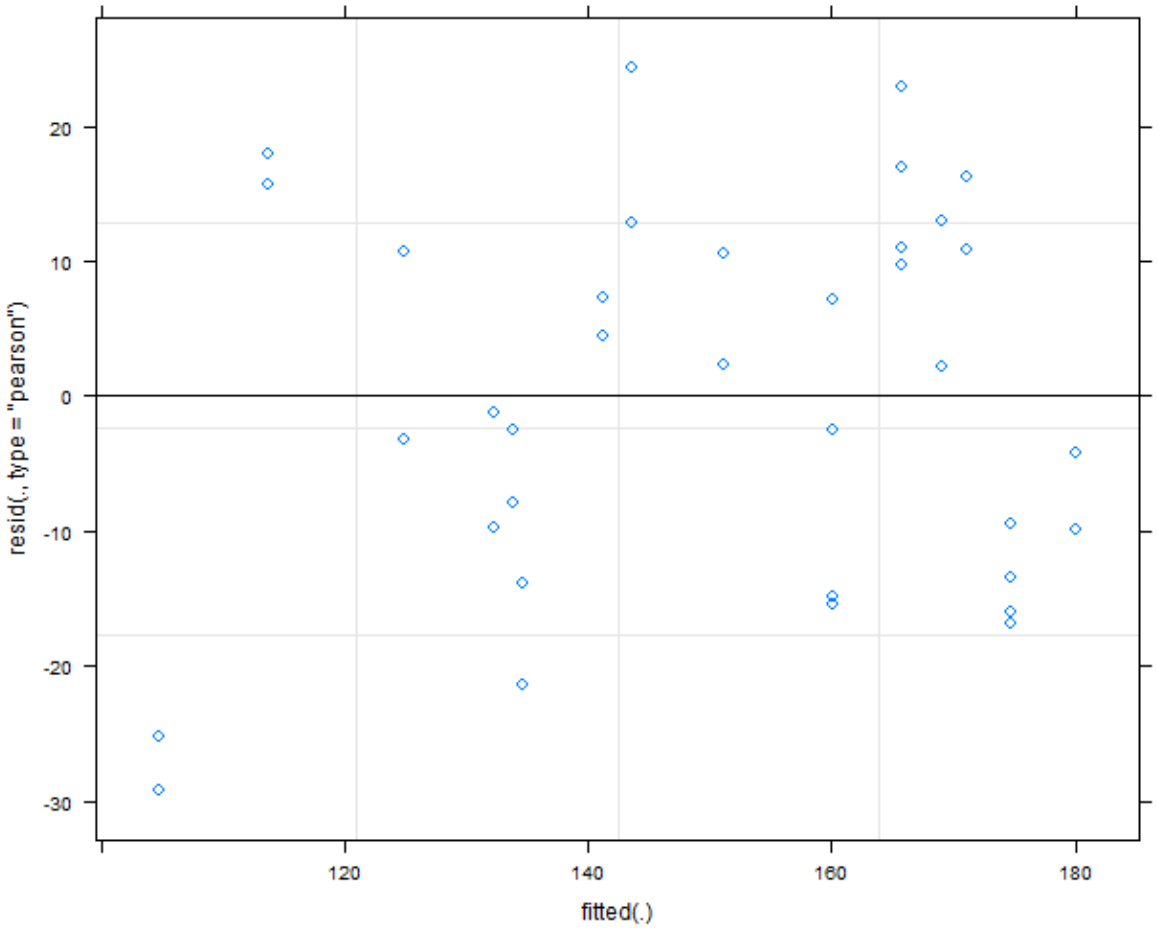
```
qqmath(model6) #check normality assumption
```




```
shapiro.test(resid(model6))
```

```
##  Shapiro-Wilk normality test
## data:  resid(model6)
## W = 0.96772, p-value = 0.3665
```

```
plot(model6, results="hide", fig.show='hide') #plot residuals vs fitted values to check for unequal variance
```




```
#Test for significance of random predictor male ID
model6.1<-lmer(VAP ~  as.factor(YEAR) +SOCIAL.STATUS+ (1|WEEK)
,data=CHANGESTOSDVAP)
anova(model6,model6.1)

## refitting model(s) with ML (instead of REML)

## Data: CHANGESTOSDVAP
## Models:
## ..1: VAP ~ as.factor(YEAR) + SOCIAL.STATUS + (1 | WEEK)
## object: VAP ~ as.factor(YEAR) + SOCIAL.STATUS + (1 | WEEK) + (1 |
MALE.ID)
##        Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
## ..1     6 341.67 351.17 -164.84   329.67
## object  7 331.43 342.51 -158.72   317.43 12.242      1  0.0004672 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Model that compares mean VAP for males that are S (stage 1) that become SS (stage 2).

```
CHANGESTOSSVAP<-read.table(file="
CHANGE_VAP_S_TO_SS.csv",header=T,row.names=NULL,sep=",")#load data frame


model7<-lmer(VAP ~  as.factor(YEAR) +SOCIAL.STATUS+ (1|WEEK) + (1|MALE.ID)
,data=CHANGESTOSSVAP)
summary(model7)
```

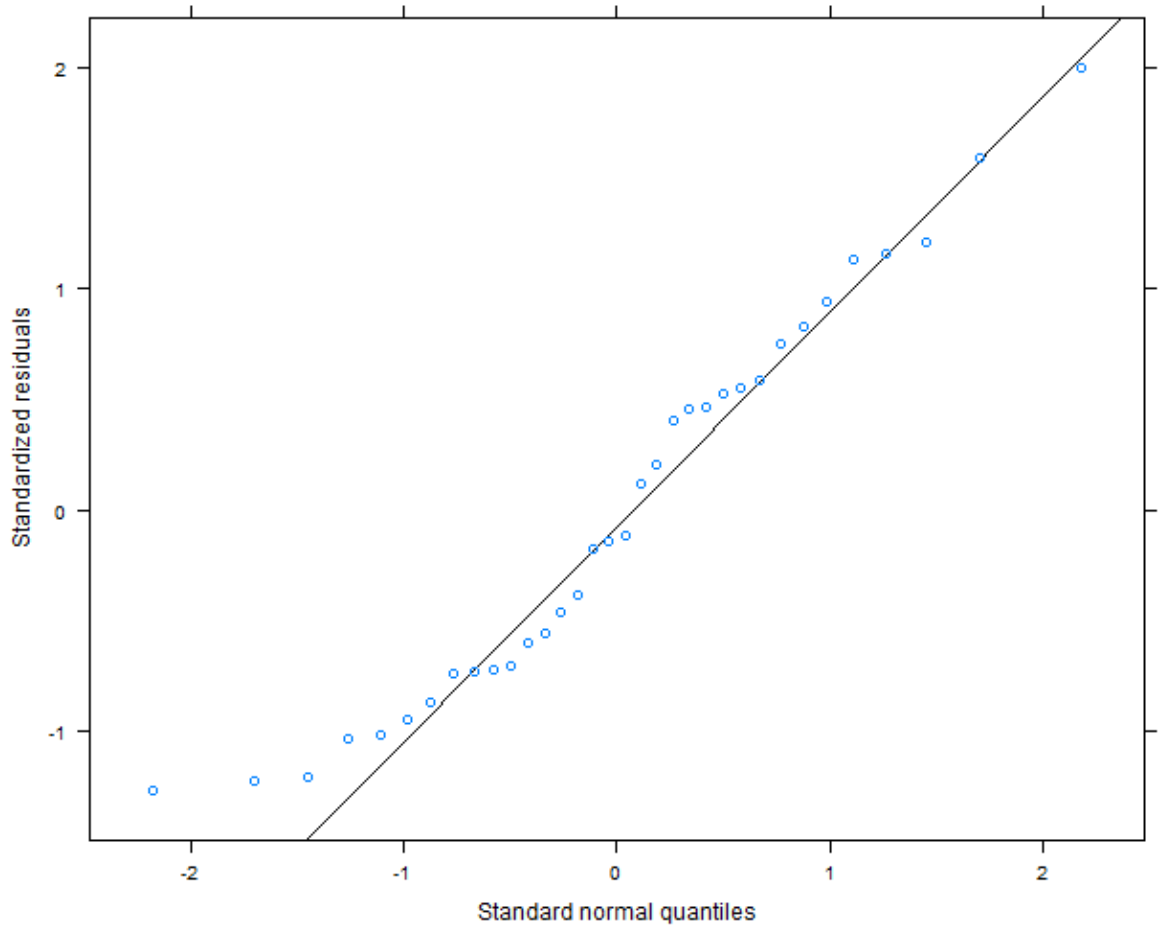
```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula:
## VAP ~ as.factor(YEAR) + SOCIAL.STATUS + (1 | WEEK) + (1 | MALE.ID)
##    Data: CHANGESTOSSVAP
##
## REML criterion at convergence: 261.2
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.2715 -0.7319 -0.1309  0.5791  2.0023
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MALE.ID  (Intercept) 115.7    10.76
##  WEEK     (Intercept)   0.0     0.00
##  Residual             206.0    14.35
## Number of obs: 34, groups:  MALE.ID, 9; WEEK, 5
##
## Fixed effects:
##                     Estimate Std. Error      df t value Pr(>|t|)
## (Intercept)          162.469      7.867   7.132  20.652 1.26e-07 ***
## as.factor(YEAR)2014   11.205     10.646   5.986   1.053    0.333
## as.factor(YEAR)2015   -1.153     10.646   5.986  -0.108    0.917
## SOCIAL.STATUSSS       -2.322      4.960  24.301  -0.468    0.644
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) a.(YEAR)2014 a.(YEAR)2015
## a.(YEAR)2014 -0.658
## a.(YEAR)2015 -0.673  0.491
## SOCIAL.STAT  -0.315 -0.024        0.024
```

```
confint(model7, level=0.95, method="Wald",oldNames=F) #generate 95%CI
using Wald method
```

```
##                               2.5 %     97.5 %
## sd_(Intercept)|MALE.ID           NA         NA
## sd_(Intercept)|WEEK              NA         NA
## sigma                            NA         NA
## (Intercept)             147.050721 177.888267
## as.factor(YEAR)2014      -9.659388  32.070375
```

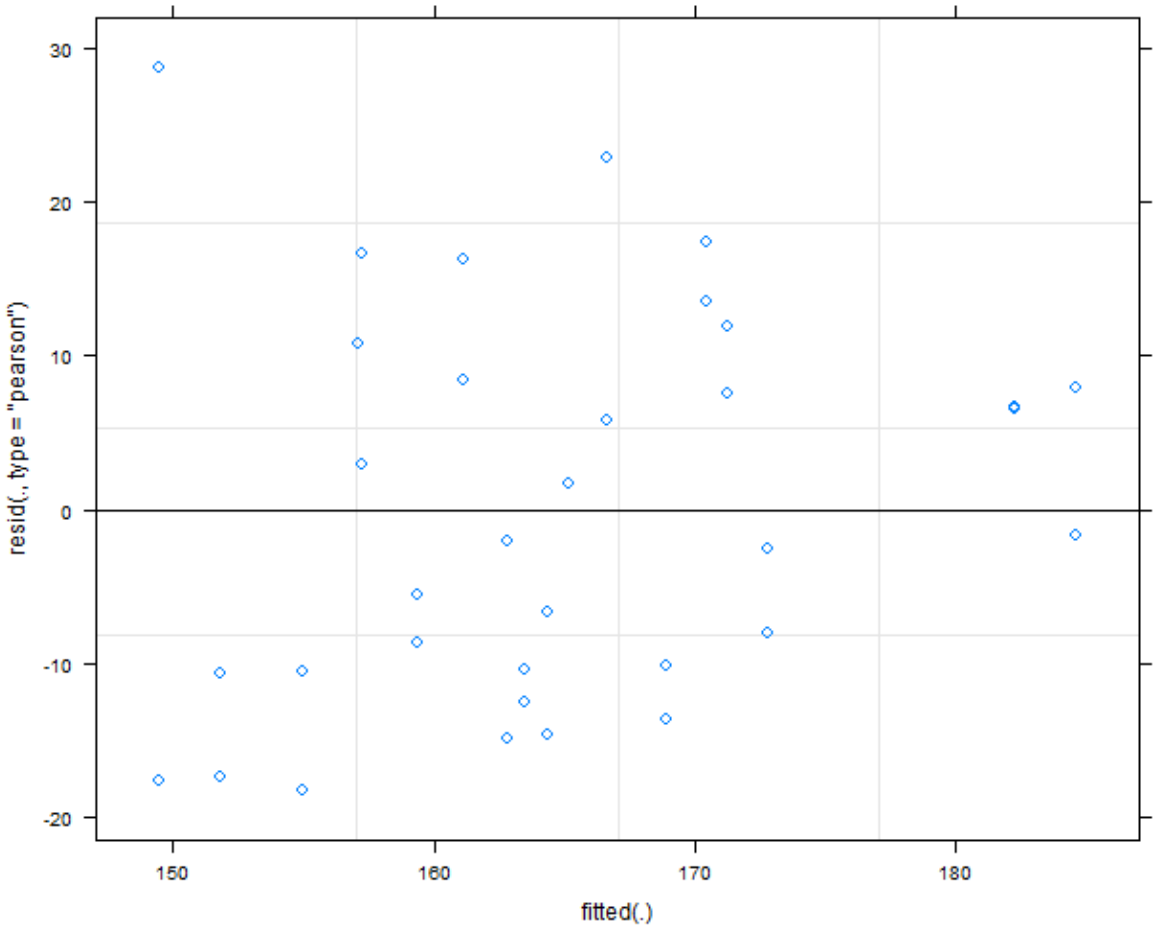
```
## as.factor(YEAR)2015    -22.017870  19.711893
## SOCIAL.STATUSSS        -12.043283   7.398641
```

```
qqmath(model7) #check normality assumption
```




```
shapiro.test(resid(model7))
##  Shapiro-Wilk normality test
## data:  resid(model7)
## W = 0.95274, p-value = 0.1483
```

```
plot(model7, results="hide", fig.show='hide') #plot residuals vs fitted
values to check for unequal variance
```




```
#Test for significance of random predictor male ID
model7.1<-lmer(VAP ~  as.factor(YEAR) +SOCIAL.STATUS+ (1|WEEK)
,data=CHANGESTOSSVAP)
anova(model7,model7.1)

## refitting model(s) with ML (instead of REML)

## Data: CHANGESTOSSVAP
## Models:
## ..1: VAP ~ as.factor(YEAR) + SOCIAL.STATUS + (1 | WEEK)
## object: VAP ~ as.factor(YEAR) + SOCIAL.STATUS + (1 | WEEK) + (1 |
MALE.ID)
##        Df    AIC    BIC  logLik deviance Chisq Chi Df Pr(>Chisq)
## ..1     6 297.44 306.60 -142.72   285.44
## object  7 297.19 307.88 -141.60   283.19 2.252      1     0.1334
```

Models that compare sperm counts for males that are D (stage 1) that become DS (stage 2).

```
CHANGEDTODS<-read.table(file="
CHANGE_COUNT_D_TO_DS.csv",header=T,row.names=NULL,sep=",")#Load data frame


mod4<-glmer(COUNTRAW ~ as.factor(YEAR) + SOCIAL.STATUS  + (1|WEEK)
+  (1|MALE.ID) ,data=CHANGEDTODS,family="poisson")
overdisp.glmer(mod4)# check for over/under-dispersion

## Residual deviance: 267.048 on 14 degrees of freedom (ratio: 19.075)

dispersionDS<-1:length(CHANGEDTODS$YEAR)# disperion parameter for
overdispersed models


mod4.a<-glmer(COUNTRAW ~ as.factor(YEAR) + SOCIAL.STATUS  + (1|WEEK)
+  (1|MALE.ID) +(1|dispersionDS),data=CHANGEDTODS,family="poisson")#model
overdispersed so added dispersion parameter
summary(mod4.a)

## Generalized linear mixed model fit by maximum likelihood (Laplace
##   Approximation) [glmerMod]
##  Family: poisson  ( log )
## Formula: COUNTRAW ~ as.factor(YEAR) + SOCIAL.STATUS + (1 | WEEK) +
## (1 | MALE.ID) + (1 | dispersionDS)
##    Data: CHANGEDTODS
##
##      AIC      BIC   logLik deviance df.resid
##    257.7    264.7   -121.8    243.7       13
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -0.29009 -0.18281 -0.00837  0.11728  0.32824
##
## Random effects:
##  Groups       Name        Variance Std.Dev.
##  dispersionDS (Intercept) 0.075765 0.27525
##  MALE.ID      (Intercept) 0.027658 0.16631
##  WEEK         (Intercept) 0.003024 0.05499
## Number of obs: 20, groups:  dispersionDS, 20; MALE.ID, 10; WEEK, 5
##
## Fixed effects:
##                     Estimate Std. Error z value Pr(>|z|)
## (Intercept)          6.06827    0.25941  23.393   <2e-16 ***
## as.factor(YEAR)2014 -0.52755    0.27652  -1.908   0.0564 .
## as.factor(YEAR)2015  0.13801    0.29012   0.476   0.6343
## SOCIAL.STATUSDS     -0.09749    0.12576  -0.775   0.4382
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
```

```
##              (Intr) a.(YEAR)2014 a.(YEAR)2015
## a.(YEAR)2014 -0.876
## a.(YEAR)2015 -0.826  0.776
## SOCIAL.STAT  -0.237 -0.006       -0.004
```

```
overdisp.glmer(mod4.a)# check for over/under-dispersion
```

```
## Residual deviance: 0.604 on 13 degrees of freedom (ratio: 0.046)
```

```
confint(mod4.a, level=0.95, method="Wald",oldNames=F) #generate 95%CI
using Wald method
```

```
##                                    2.5 %     97.5 %
## sd_(Intercept)|dispersionDS           NA         NA
## sd_(Intercept)|MALE.ID                NA         NA
## sd_(Intercept)|WEEK                   NA         NA
## (Intercept)                    5.5598439 6.57669612
## as.factor(YEAR)2014           -1.0695157 0.01441253
## as.factor(YEAR)2015           -0.4306062 0.70663518
## SOCIAL.STATUSDS               -0.3439648 0.14898771
```

```
#Test for significance of random predictor male ID
mod4.1<-glmer(COUNTRAW ~ as.factor(YEAR) + SOCIAL.STATUS  +
(1|WEEK)+(1|dispersionDS) ,data=CHANGEDTODS,family="poisson")
anova(mod4.a,mod4.1)
```

```
## Data: CHANGEDTODS
## Models:
## mod4.1: COUNTRAW ~ as.factor(YEAR) + SOCIAL.STATUS + (1 | WEEK) + (1 |
## mod4.1:     dispersionDS)
## mod4.a: COUNTRAW ~ as.factor(YEAR) + SOCIAL.STATUS + (1 | WEEK) + (1 |
## mod4.a:     MALE.ID) + (1 | dispersionDS)
##        Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
## mod4.1  6 256.18 262.15 -122.09   244.18
## mod4.a  7 257.69 264.66 -121.84   243.69 0.4887      1     0.4845
```

Models that compare sperm counts for males that are D(stage 1) that become DD (stage 2).

```
CHANGEDTODD<-read.table(file="
CHANGE_COUNT_D_TO_DD.csv",header=T,row.names=NULL,sep=",")#Load data frame


mod5<-glmer(COUNTRAW ~ as.factor(YEAR) + SOCIAL.STATUS  + (1|WEEK)
+  (1|MALE.ID) ,data=CHANGEDTODD,family="poisson")
overdisp.glmer(mod5)# check for over/under-dispersion

## Residual deviance: 228.037 on 14 degrees of freedom (ratio: 16.288)

dispersionDD<-1:length(CHANGEDTODD$YEAR)# disperion parameter for
overdispersed models


mod5.a<-glmer(COUNTRAW ~ as.factor(YEAR) + SOCIAL.STATUS  + (1|WEEK)
+  (1|MALE.ID) +(1|dispersionDD),data=CHANGEDTODD,family="poisson")#model
overdispersed so added dispersion parameter
summary(mod5.a)

## Generalized linear mixed model fit by maximum likelihood (Laplace
##   Approximation) [glmerMod]
##  Family: poisson  ( log )
## Formula: COUNTRAW ~ as.factor(YEAR) + SOCIAL.STATUS + (1 | WEEK) +
## (1 | MALE.ID) + (1 | dispersionDD)
##    Data: CHANGEDTODD
##
##      AIC      BIC   logLik deviance df.resid
##    247.3    254.3   -116.7    233.3       13
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -0.61632 -0.11273  0.03231  0.10608  0.27940
##
## Random effects:
##  Groups       Name        Variance  Std.Dev.
##  dispersionDD (Intercept) 6.514e-02 2.552e-01
##  MALE.ID      (Intercept) 4.505e-09 6.712e-05
##  WEEK         (Intercept) 2.874e-03 5.361e-02
## Number of obs: 20, groups:  dispersionDD, 20; MALE.ID, 10; WEEK, 5
##
## Fixed effects:
##                      Estimate Std. Error z value Pr(>|z|)
## (Intercept)           5.6523     0.1598   35.37   <2e-16 ***
## as.factor(YEAR)2014   0.2112     0.1681    1.26    0.209
## as.factor(YEAR)2015   0.1888     0.1953    0.97    0.334
## SOCIAL.STATUSDD      -0.1647     0.1170   -1.41    0.159
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##              (Intr) a.(YEAR)2014 a.(YEAR)2015
## a.(YEAR)2014 -0.800
```

```
## a.(YEAR)2015 -0.753  0.713
## SOCIAL.STAT  -0.368  0.002        0.005
```

```
confint(mod5.a, level=0.95, method="Wald",oldNames=F) #generate 95%CI
using Wald method
```

```
##                                    2.5 %     97.5 %
## sd_(Intercept)|dispersionDD           NA         NA
## sd_(Intercept)|MALE.ID                NA         NA
## sd_(Intercept)|WEEK                   NA         NA
## (Intercept)                    5.3390749 5.96545984
## as.factor(YEAR)2014           -0.1182952 0.54076135
## as.factor(YEAR)2015           -0.1940943 0.57161437
## SOCIAL.STATUSDD               -0.3940623 0.06472596
```

```
#Test for significance of random predictor male ID
mod5.1<-glmer(COUNTRAW ~ as.factor(YEAR) + SOCIAL.STATUS  +
(1|WEEK)+(1|dispersionDD) ,data=CHANGEDTODD,family="poisson")
anova(mod5.a,mod5.1)
```

```
## Data: CHANGEDTODD
## Models:
## mod5.1: COUNTRAW ~ as.factor(YEAR) + SOCIAL.STATUS + (1 | WEEK) + (1 |
## mod5.1:     dispersionDD)
## mod5.a: COUNTRAW ~ as.factor(YEAR) + SOCIAL.STATUS + (1 | WEEK) + (1 |
## mod5.a:     MALE.ID) + (1 | dispersionDD)
##        Df    AIC    BIC  logLik deviance Chisq Chi Df Pr(>Chisq)
## mod5.1  6 245.33 251.31 -116.67   233.33
## mod5.a  7 247.33 254.30 -116.67   233.33     0      1          1
```

Models that compare sperm counts for males that are S (stage 1) that become SD (stage 2).

```
CHANGESTOSD<-read.table(file="
CHANGE_COUNT_S_TO_SD.csv",header=T,row.names=NULL,sep=",")#Load data frame


mod6<-glmer(COUNTRAW ~ as.factor(YEAR) + SOCIAL.STATUS  + (1|WEEK)
+  (1|MALE.ID) ,data=CHANGESTOSD,family="poisson")
overdisp.glmer(mod6)# check for over/under-dispersion

## Residual deviance: 435.473 on 12 degrees of freedom (ratio: 36.289)

dispersionSD<-1:length(CHANGESTOSD$YEAR)# disperion parameter for
overdispersed models


mod6.a<-glmer(COUNTRAW ~ as.factor(YEAR) + SOCIAL.STATUS  + (1|WEEK)
+  (1|MALE.ID)+(1|dispersionSD),data=CHANGESTOSD,family="poisson")#model
overdispersed so added dispersion parameter
summary(mod6.a)

## Generalized linear mixed model fit by maximum likelihood (Laplace
##   Approximation) [glmerMod]
##  Family: poisson  ( log )
## Formula: COUNTRAW ~ as.factor(YEAR) + SOCIAL.STATUS + (1 | WEEK) +
## (1 | MALE.ID) + (1 | dispersionSD)
##    Data: CHANGESTOSD
##
##      AIC      BIC   logLik deviance df.resid
##    234.5    240.7   -110.2    220.5       11
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -0.36301 -0.10226  0.00885  0.07905  0.30047
##
## Random effects:
##  Groups       Name        Variance  Std.Dev.
##  dispersionSD (Intercept) 6.485e-02 2.547e-01
##  MALE.ID      (Intercept) 1.024e-10 1.012e-05
##  WEEK         (Intercept) 2.426e-03 4.925e-02
## Number of obs: 18, groups:  dispersionSD, 18; MALE.ID, 9; WEEK, 5
##
## Fixed effects:
##                      Estimate Std. Error z value Pr(>|z|)
## (Intercept)            6.4345     0.1256   51.23  < 2e-16 ***
## as.factor(YEAR)2014  -0.5832     0.1519   -3.84 0.000124 ***
## as.factor(YEAR)2015  -0.2383     0.1515   -1.57 0.115878
## SOCIAL.STATUSSD      -0.2379     0.1224   -1.94 0.051876 .
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##              (Intr) a.(YEAR)2014 a.(YEAR)2015
## a.(YEAR)2014 -0.594
```

```
## a.(YEAR)2015 -0.605  0.490
## SOCIAL.STAT  -0.487  0.005        0.001
```

```
overdisp.glmer(mod6.a)# check for over/under-dispersion
```

```
## Residual deviance: 0.605 on 11 degrees of freedom (ratio: 0.055)
```

```
confint(mod6.a, level=0.95, method="Wald",oldNames=F) #generate 95%CI
using Wald method
```

```
##                                    2.5 %       97.5 %
## sd_(Intercept)|dispersionSD           NA           NA
## sd_(Intercept)|MALE.ID                NA           NA
## sd_(Intercept)|WEEK                   NA           NA
## (Intercept)                    6.1882898  6.680617265
## as.factor(YEAR)2014           -0.8809774 -0.285446552
## as.factor(YEAR)2015           -0.5352977  0.058746261
## SOCIAL.STATUSSD               -0.4777961  0.001933525
```

```
#Test for significance of random predictor male ID
mod6.1<-glmer(COUNTRAW ~ as.factor(YEAR) + SOCIAL.STATUS  +
+(1|dispersionSD)+(1|WEEK) ,data=CHANGESTOSD,family="poisson")
anova(mod6.a,mod6.1)
```

```
## Data: CHANGESTOSD
## Models:
## mod6.1: COUNTRAW ~ as.factor(YEAR) + SOCIAL.STATUS + +(1 |
dispersionSD) +
## mod6.1:     (1 | WEEK)
## mod6.a: COUNTRAW ~ as.factor(YEAR) + SOCIAL.STATUS + (1 | WEEK) + (1 |
## mod6.a:     MALE.ID) + (1 | dispersionSD)
##        Df    AIC    BIC  logLik deviance Chisq Chi Df Pr(>Chisq)
## mod6.1  6 232.47 237.81 -110.23   220.47
## mod6.a  7 234.47 240.70 -110.23   220.47     0      1          1
```

Models that compare sperm counts for males that are S (stage 1) that become SS (stage 2).

```
CHANGESTOSS<-read.table(file="
CHANGE_COUNT_S_TO_SS.csv",header=T,row.names=NULL,sep=",")#Load data frame


mod7<-glmer(COUNTRAW ~ as.factor(YEAR) + SOCIAL.STATUS  + (1|WEEK)
+  (1|MALE.ID) ,data=CHANGESTOSS,family="poisson")
overdisp.glmer(mod7)# check for over/under-dispersion

## Residual deviance: 406.822 on 12 degrees of freedom (ratio: 33.902)

dispersionSS<-1:length(CHANGESTOSS$YEAR)# disperion parameter for
overdispersed models


mod7.a<-glmer(COUNTRAW ~ as.factor(YEAR) + SOCIAL.STATUS  + (1|WEEK)
+  (1|MALE.ID) + (1|dispersionSS),data=CHANGESTOSS,family="poisson")#model
overdispersed so added dispersion parameter
summary(mod7.a)

## Generalized linear mixed model fit by maximum likelihood (Laplace
##   Approximation) [glmerMod]
##  Family: poisson  ( log )
## Formula: COUNTRAW ~ as.factor(YEAR) + SOCIAL.STATUS + (1 | WEEK) + (1
|
##     MALE.ID) + (1 | dispersionSS)
##    Data: CHANGESTOSS
##
##      AIC      BIC   logLik deviance df.resid
##    238.2    244.4   -112.1    224.2       11
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -0.31801 -0.14250 -0.03193  0.05426  0.25890
##
## Random effects:
##  Groups       Name        Variance  Std.Dev.
##  dispersionSS (Intercept) 7.771e-02 2.788e-01
##  MALE.ID      (Intercept) 0.000e+00 0.000e+00
##  WEEK         (Intercept) 3.521e-10 1.876e-05
## Number of obs: 18, groups:  dispersionSS, 18; MALE.ID, 10; WEEK, 5
##
## Fixed effects:
##                     Estimate Std. Error z value Pr(>|z|)
## (Intercept)          6.35425    0.13301   47.77  < 2e-16 ***
## as.factor(YEAR)2014 -0.58610    0.16343   -3.59 0.000335 ***
## as.factor(YEAR)2015 -0.39093    0.16313   -2.40 0.016557 *
## SOCIAL.STATUSSS      0.09117    0.13346    0.68 0.494526
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##              (Intr) a.(YEAR)2014 a.(YEAR)2015
```

```
## a.(YEAR)2014 -0.608
## a.(YEAR)2015 -0.611  0.497
## SOCIAL.STAT  -0.501 -0.003        0.000
```

```
confint(mod7.a, level=0.95, method="Wald",oldNames=F) #generate 95%CI
using Wald method
```

```
##                                    2.5 %      97.5 %
## sd_(Intercept)|dispersionSS           NA          NA
## sd_(Intercept)|MALE.ID                NA          NA
## sd_(Intercept)|WEEK                   NA          NA
## (Intercept)                    6.0935652  6.61494461
## as.factor(YEAR)2014           -0.9064065 -0.26578688
## as.factor(YEAR)2015           -0.7106708 -0.07119744
## SOCIAL.STATUSSS               -0.1704098  0.35275361
```

```
#Test for significance of random predictor male ID
mod7.1<-glmer(COUNTRAW ~ as.factor(YEAR) + SOCIAL.STATUS  + (1|WEEK)+
(1|dispersionSS) ,data=CHANGESTOSS,family="poisson")
anova(mod7.a,mod7.1)
```

```
## Data: CHANGESTOSS
## Models:
## mod7.1: COUNTRAW ~ as.factor(YEAR) + SOCIAL.STATUS + (1 | WEEK) + (1 |
## mod7.1:     dispersionSS)
## mod7.a: COUNTRAW ~ as.factor(YEAR) + SOCIAL.STATUS + (1 | WEEK) + (1 |
## mod7.a:     MALE.ID) + (1 | dispersionSS)
##        Df    AIC    BIC logLik deviance Chisq Chi Df Pr(>Chisq)
## mod7.1  6 236.21 241.55 -112.1   224.21
## mod7.a  7 238.21 244.44 -112.1   224.21     0      1     0.9998
```

Model that tests for an interaction effect between social phenotype and experimental stage with VAP as response variable.

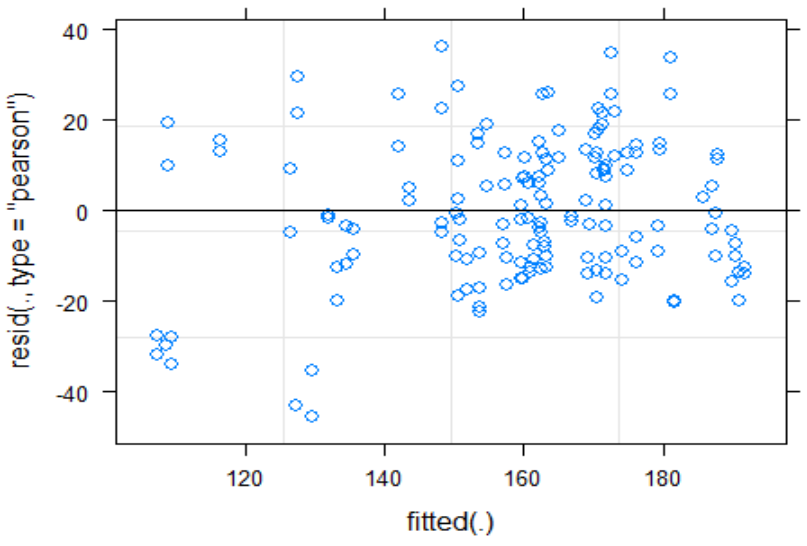
```
TESTFORINTERACTIONVAP<-read.table(file="CHANGESSSPLOT.csv",header=T,row.names=NULL,sep=",")#load data frame


modelINT<-lmer(VAP ~ as.factor(YEAR) + GROUP + as.factor(STAGE) + GROUP:as.factor(STAGE) + (1|WEEK) + (1|MALE.ID) ,data=TESTFORINTERACTIONVAP)
summary(modelINT)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula:
## VAP ~ as.factor(YEAR) + GROUP + as.factor(STAGE) + GROUP:as.factor(STAGE) +
##     (1 | WEEK) + (1 | MALE.ID)
##    Data: TESTFORINTERACTIONVAP
##
## REML criterion at convergence: 1302.9
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -2.52976 -0.59331 -0.08737  0.65619  2.01827
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MALE.ID  (Intercept) 354.15   18.819
##  WEEK     (Intercept)  83.96    9.163
##  Residual             325.72   18.048
## Number of obs: 151, groups:  MALE.ID, 38; WEEK, 5
##
## Fixed effects:
##                       Estimate Std. Error       df t value Pr(>|t|)
## (Intercept)           142.0250    10.7297  23.3900  13.237 2.36e-12  ***
## as.factor(YEAR)2014  22.5507     9.0120  31.5900   2.502   0.0177    *
## as.factor(YEAR)2015  15.9919     9.5815  31.5600   1.669   0.1050
## GROUPD-S             -1.8400    10.1686  42.4800  -0.181   0.8573
## GROUPS-D             1.2965    10.5956  42.6500   0.122   0.9032
## GROUPS-S            12.2020    10.5956  42.6500   1.152   0.2559
## as.factor(STAGE)2   -0.2957     5.7216 108.7500  -0.052   0.9589
## GROUPD-S: (STAGE)2  18.9140     8.1476 108.3200   2.321   0.0221 *
## GROUPS-D: (STAGE)2  -8.6932     8.3023 108.1200  -1.047   0.2974
## GROUPS-S: (STAGE)2  -0.8765     8.3023 108.1200  -0.106   0.9161
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##              (Intr) a.(YEAR)2014 a.(YEAR)2015 GROUPD-S GROUPS-D GROUPS-S
## a.(YEAR)2014 -0.597
## a.(YEAR)2015 -0.519  0.592
## GROUPD-S     -0.474  0.000        0.000
## GROUPS-D     -0.528  0.132        0.059        0.480
```

```
## GROUPS-S      -0.528  0.132        0.059        0.480    0.488
## as.(STAGE)2   -0.275  0.012        0.018        0.280    0.275    0.275
## GROUPD-S:.(    0.192 -0.005       -0.015       -0.393   -0.192   -0.192
## GROUPS-D:.(    0.190 -0.008       -0.012       -0.193   -0.396   -0.190
## GROUPS-S:.(    0.190 -0.008       -0.012       -0.193   -0.190   -0.396
##               a.(STA GROUPD-S: GROUPS-D:
## a.(YEAR)2014
## a.(YEAR)2015
## GROUPD-S
## GROUPS-D
## GROUPS-S
## as.(STAGE)2
## GROUPD-S:.(   -0.702
## GROUPS-D:.(   -0.689  0.484
## GROUPS-S:.(   -0.689  0.484     0.475
```

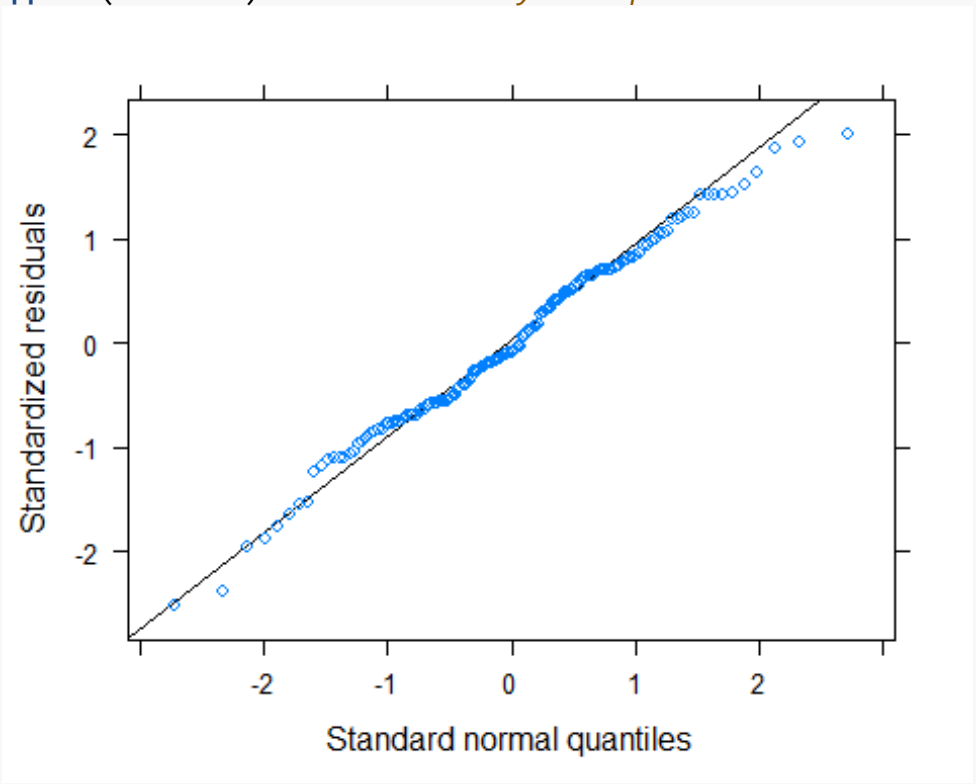
```
confint(modelINT, level=0.95, method="Wald",oldNames=F) #generate 95%CI us
ing Wald method
```

```
##                                    2.5 %     97.5 %
## sd_(Intercept)|MALE.ID                NA         NA
## sd_(Intercept)|WEEK                   NA         NA
## sigma                                 NA         NA
## (Intercept)                  120.995106 163.054952
## as.factor(YEAR)2014            4.887503  40.213940
## as.factor(YEAR)2015           -2.787519  34.771282
## GROUPD-S                     -21.770180  18.090180
## GROUPS-D                     -19.470444  22.063425
## GROUPS-S                      -8.564888  32.968981
## as.factor(STAGE)2            -11.509833  10.918476
## GROUPD-S:as.factor(STAGE)2     2.945134  34.882962
## GROUPS-D:as.factor(STAGE)2   -24.965396   7.578975
## GROUPS-S:as.factor(STAGE)2   -17.148729  15.395642
```

```
plot(modelINT, results="hide", fig.show='hide') #plot residuals vs fitted
values to check for unequal variance
```



```
qqmath(modelINT) #check normality assumption
```




```
shapiro.test(resid(modelINT))
##  Shapiro-Wilk normality test
## data:  resid(modelINT)
## W = 0.99013, p-value = 0.3721

#Test for significance of random predictor male ID
modelINT.1<-lmer(VAP ~ as.factor(YEAR) + GROUP*as.factor(STAGE) + (1|WEEK)
,data=TESTFORINTERACTIONVAP)
anova(modelINT,modelINT.1)

## refitting model(s) with ML (instead of REML)

## Data: TESTFORINTERACTIONVAP
## Models:
## ..1: VAP ~ as.factor(YEAR) + GROUP * as.factor(STAGE) + (1 | WEEK)
## object: VAP ~ as.factor(YEAR) + GROUP + as.factor(STAGE) + GROUP:as.fac
tor(STAGE) +
## object:     (1 | WEEK) + (1 | MALE.ID)
##        Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
## ..1    12 1422.7 1458.9 -699.37   1398.7
## object 13 1385.5 1424.8 -679.76   1359.5 39.212      1  3.802e-10 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

#Test for significance of overall interaction term
modelINT.2<-lmer(VAP ~ as.factor(YEAR) + GROUP + as.factor(STAGE) + (1|WEE
K)+ (1|MALE.ID) ,data=TESTFORINTERACTIONVAP)
anova(modelINT,modelINT.2)
```

```
## refitting model(s) with ML (instead of REML)

## Data: TESTFORINTERACTIONVAP
## Models:
## ..1: VAP ~ as.factor(YEAR) + GROUP + as.factor(STAGE) + (1 | WEEK) +
## ..1:     (1 | MALE.ID)
## object: VAP ~ as.factor(YEAR) + GROUP + as.factor(STAGE) + GROUP:as.fac
tor(STAGE) +
## object:     (1 | WEEK) + (1 | MALE.ID)
##        Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
## ..1    10 1391.3 1421.5 -685.67   1371.3
## object 13 1385.5 1424.8 -679.76   1359.5 11.806      3   0.008077 **
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Model that tests for an interaction effect between social phenotype and experimental stage with sperm concentration as response variable.

```
TESTFORINTERACTIONCOUNT<-read.table(file="CHANGESSPLOT2.csv",header=T,row.names=NULL,sep=",")#load data frame
modelINT2<-glmer(COUNTB ~ as.factor(YEAR) + GROUP*as.factor(STAGE)  +(1|WEEK)+ (1|MALE.ID) ,data=TESTFORINTERACTIONCOUNT, family="poisson")
overdisp.glmer(modelINT2)# check for over/under-dispersion

## Residual deviance: 1337.064 on 64 degrees of freedom (ratio: 20.892)


#Model failed to converge with the addition of dispersion parameter so removing non-significant random term (1|WEEK)

#Test for significance of random predictor WEEK
modelINT2.1<-glmer(COUNTB ~ as.factor(YEAR) + GROUP*as.factor(STAGE)  + (1|MALE.ID) ,data=TESTFORINTERACTIONCOUNT, family="poisson")
anova(modelINT2,modelINT2.1)

## Data: TESTFORINTERACTIONCOUNT
## Models:
## modelINT2.1: COUNTB ~ as.factor(YEAR) + GROUP * as.factor(STAGE) + (1 | MALE.ID)
## modelINT2: COUNTB ~ as.factor(YEAR) + GROUP * as.factor(STAGE) + (1 | WEEK) +
## modelINT2:     (1 | MALE.ID)
##             Df    AIC    BIC  logLik deviance Chisq Chi Df Pr(>Chisq)
## modelINT2.1 11 2140.8 2166.4 -1059.4   2118.8
## modelINT2   12 2142.8 2170.8 -1059.4   2118.8     0      1          1

dispersionINT2<-1:length(TESTFORINTERACTIONCOUNT$YEAR)# dispersion parameter for overdispersed model

#running model with dispersion parameter and (1|WEEK) removed
modINT2.2<-glmer(COUNTB ~ as.factor(YEAR) + GROUP*as.factor(STAGE)  +  (1|MALE.ID) +(1|dispersionINT2),data=TESTFORINTERACTIONCOUNT,family="poisson")#model overdispersed so added dispersion parameter
overdisp.glmer(modINT2.2)# check for over/under-dispersion

## Residual deviance: 2.077 on 64 degrees of freedom (ratio: 0.032)

summary(modINT2.2)

## Generalized linear mixed model fit by maximum likelihood (Laplace
##   Approximation) [glmerMod]
##  Family: poisson  ( log )
## Formula:
## COUNTB ~ as.factor(YEAR) + GROUP * as.factor(STAGE) + (1 | MALE.ID) +
##     (1 | dispersionINT2)
##    Data: TESTFORINTERACTIONCOUNT
##
##      AIC      BIC   logLik deviance df.resid
##    967.4    995.4   -471.7    943.4       64
##
```

```
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -0.39724 -0.13159 -0.00873  0.10502  0.25812
##
## Random effects:
##  Groups         Name        Variance Std.Dev.
##  dispersionINT2 (Intercept) 0.08549  0.2924
##  MALE.ID        (Intercept) 0.02114  0.1454
## Number of obs: 76, groups:  dispersionINT2, 76; MALE.ID, 38
##
## Fixed effects:
##                     Estimate Std. Error    z value Pr(>|z|)
## (Intercept)          6.04682    0.12989   46.55     < 2e-16 ***
## as.factor(YEAR)2014 -0.39715    0.10511   -3.78    0.000158 ***
## as.factor(YEAR)2015 -0.11287    0.10997   -1.03    0.304735
## GROUPD-S             0.03616    0.14820    0.24    0.807235
## GROUPS-D             0.27538    0.15256    1.81    0.071053 .
## GROUPS-S             0.14945    0.15239    0.98    0.326723
## as.factor(STAGE)2   -0.16450    0.13331   -1.23    0.217202
## GROUPD-S: (STAGE)2   0.06689    0.18849    0.35    0.722700
## GROUPS-D: (STAGE)2  -0.07374    0.19322   -0.38    0.702732
## GROUPS-S: (STAGE)2   0.26365    0.19427    1.36    0.174727
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##              (Intr) a.(YEAR)2014 a.(YEAR)2015 GROUPD-S GROUPS-D GROUPS-
S
## a.(YEAR)2014 -0.554
## a.(YEAR)2015 -0.492  0.584
## GROUPD-S     -0.572  0.003        0.001
## GROUPS-D     -0.602  0.095        0.038        0.486
## GROUPS-S     -0.605  0.099        0.041        0.486    0.494
## as.(STAGE)2  -0.512  0.000        0.002        0.449    0.436    0.436
## GROUPD-S:.(   0.363 -0.002       -0.002       -0.635   -0.308   -0.309
## GROUPS-D:.(   0.353  0.001       -0.001       -0.309   -0.631   -0.301
## GROUPS-S:.(   0.346  0.008        0.007       -0.308   -0.307   -0.634
##              a.(STA GROUPD-S: GROUPS-D:
## a.(YEAR)2014
## a.(YEAR)2015
## GROUPD-S
## GROUPS-D
## GROUPS-S
## as.(STAGE)2
## GROUPD-S:.(  -0.707
## GROUPS-D:.(  -0.690  0.488
## GROUPS-S:.(  -0.686  0.485     0.473
```

```
confint(modINT2.2,level=0.95, method="Wald",oldNames=F) #generate 95%CI us
ing Wald method
```

```
##                                      2.5 %      97.5 %
## sd_(Intercept)|dispersionINT2           NA          NA
## sd_(Intercept)|MALE.ID                  NA          NA
```

```
## (Intercept)                    5.79224548  6.30140126
## as.factor(YEAR)2014           -0.60316188 -0.19114440
## as.factor(YEAR)2015           -0.32841239  0.10267428
## GROUPD-S                      -0.25429946  0.32661757
## GROUPS-D                      -0.02361979  0.57438865
## GROUPS-S                      -0.14922277  0.44812744
## as.factor(STAGE)2             -0.42577221  0.09677437
## GROUPD-S:as.factor(STAGE)2    -0.30254368  0.43631449
## GROUPS-D:as.factor(STAGE)2    -0.45244547  0.30496596
## GROUPS-S:as.factor(STAGE)2    -0.11710279  0.64440879
```

```
#Test for significance of overall interaction effect
modINT2.3<-glmer(COUNTB ~ as.factor(YEAR) + GROUP + as.factor(STAGE)  + (1
|MALE.ID) +(1|dispersionINT2) ,data=TESTFORINTERACTIONCOUNT, family="poiss
on")
anova(modINT2.2,modINT2.3)
```

```
## Data: TESTFORINTERACTIONCOUNT
## Models:
## modINT2.3: COUNTB ~ as.factor(YEAR) + GROUP + as.factor(STAGE) + (1 | M
ALE.ID) +
## modINT2.3:     (1 | dispersionINT2)
## modINT2.2: COUNTB ~ as.factor(YEAR) + GROUP * as.factor(STAGE) + (1 | M
ALE.ID) +
## modINT2.2:     (1 | dispersionINT2)
##           Df    AIC    BIC  logLik deviance Chisq Chi Df Pr(>Chisq)
## modINT2.3  9 964.46 985.44 -473.23   946.46
## modINT2.2 12 967.41 995.38 -471.71   943.41 3.044      3     0.3849
```

```
#Test for significance of Male ID
modINT2.4<-glmer(COUNTB ~ as.factor(YEAR) + GROUP*as.factor(STAGE) +(1|dis
persionINT2) ,data=TESTFORINTERACTIONCOUNT, family="poisson")
anova(modINT2.2,modINT2.4)
```

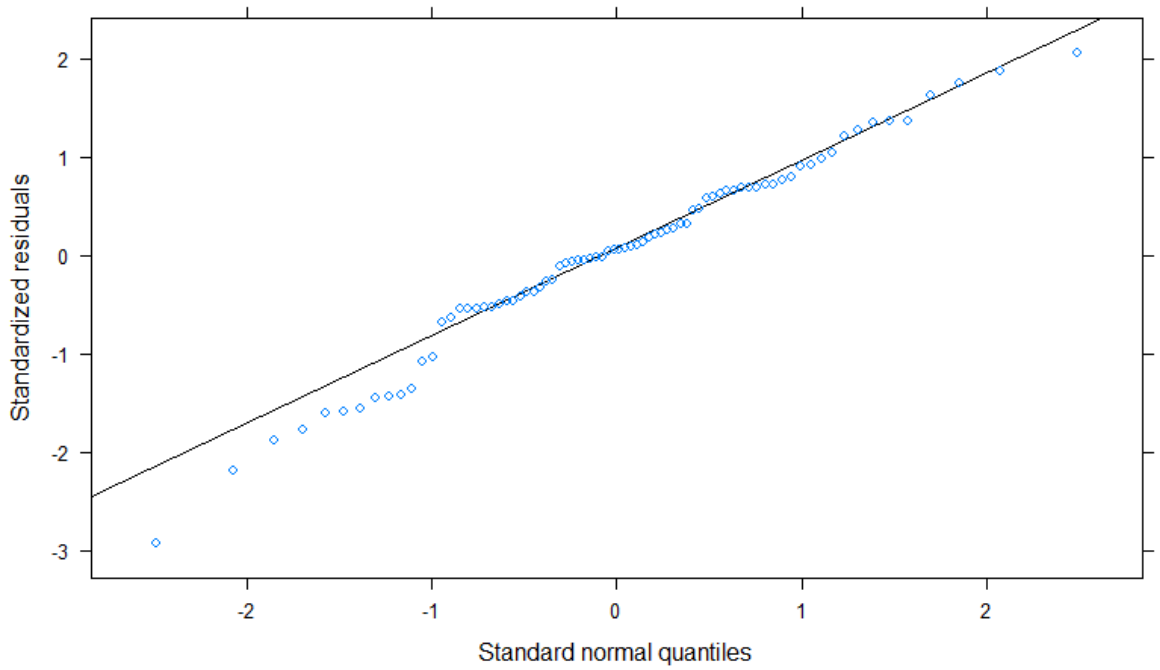
```
## Data: TESTFORINTERACTIONCOUNT
## Models:
## modINT2.4: COUNTB ~ as.factor(YEAR) + GROUP * as.factor(STAGE) + (1 | d
ispersionINT2)
## modINT2.2: COUNTB ~ as.factor(YEAR) + GROUP * as.factor(STAGE) + (1 | M
ALE.ID) +
## modINT2.2:     (1 | dispersionINT2)
##           Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
## modINT2.4 11 966.79 992.43 -472.39   944.79
## modINT2.2 12 967.41 995.38 -471.71   943.41 1.3743      1     0.2411
```

Seminal Fluid effect on sperm velocity

```
>SEMINALFLUIDSWAP<-
read.table(file="SEMINAL_FLUID_SWAP.csv",header=T,row.names=NULL,sep=",")#
import data frame

> #Social status as a fixed effect

> modswap<-lmer(Difference.in.rivals.SF ~ as.factor(YEAR)
+SOCIAL.STATUS.SF + (1|pair)
+(1|WEEK)+(1|Male.ID.sperm)+(1|Rivals.ID),data=SEMINALFLUIDSWAP)
> summary(modswap)

## Linear mixed model fit by REML
## t-tests use  Satterthwaite approximations to degrees of freedom
## ['lmerMod']
## Formula: Difference.in.rivals.SF ~ as.factor(YEAR) + SOCIAL.STATUS.SF +
(1 | pair) ## + (1 | WEEK) + (1 | Male.ID.sperm) + (1 | Rivals.ID)
##  Data: SEMINALFLUIDSWAP
##
## REML criterion at convergence: 756
##
## Scaled residuals:
##     Min       1Q   Median       3Q      Max
## -2.92965 -0.52134  0.06241  0.67950  2.06092
##
## Random effects:
## Groups        Name        Variance  Std.Dev.
## Rivals.ID     (Intercept) 1.192e-15 3.452e-08
## Male.ID.sperm (Intercept) 0.000e+00 0.000e+00
## pair          (Intercept) 0.000e+00 0.000e+00
## WEEK          (Intercept) 0.000e+00 0.000e+00
## Residual                  1.348e+03 3.672e+01
## Number of obs: 78, groups:  Rivals.ID, 42; Male.ID.sperm, 42; pair, 39;
## WEEK, 5
##
## Fixed effects:
##                     Estimate Std. Error      df t value Pr(>|t|)
## (Intercept)          -24.387      8.863  74.000  -2.752 0.007455 **
## as.factor(YEAR)2014    3.549     10.168  74.000   0.349 0.728053
## as.factor(YEAR)2015    5.793     10.837  74.000   0.535 0.594545
## SS.SFSubdominant      31.421      8.314  74.000   3.779 0.000316 ***
## ---
## Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
##
## Correlation of Fixed Effects:
##             (Intr) a.(YEAR)2014 a.(YEAR)2015
## a.(YEAR)2014 -0.680
## a.(YEAR)2015 -0.638  0.556
## SS.SFSbdmnn  -0.469  0.000        0.000
> confint(modswap, level=0.95, method="Wald",oldNames=F) #generate 95%CI
using Wald method
##                                      2.5 %    97.5 %
## (Intercept)                       -41.75825   -7.015507
```

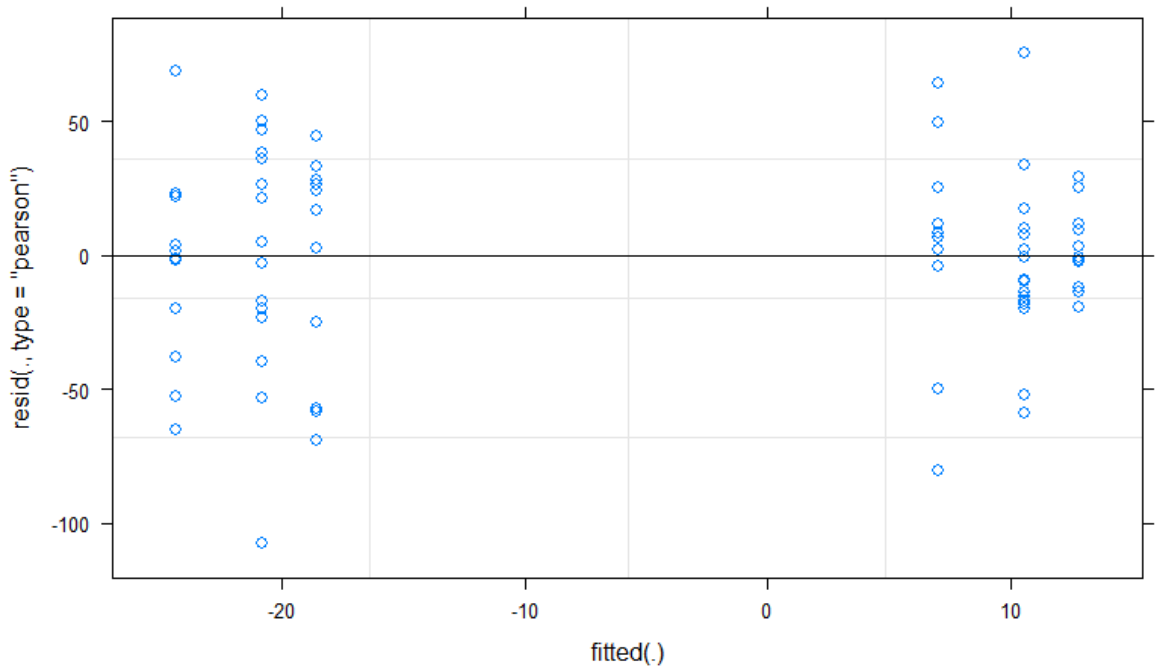
```
## as.factor(YEAR)2014                  -16.38065   23.478988
## as.factor(YEAR)2015                  -15.44683   27.033191
## SS.SFSubdominant                      15.12494   47.716517
```

```
> qqmath(modswap)
```




```
> shapiro.test(resid(modswap))

##    Shapiro-Wilk normality test
##
## data:  resid(modswap)
## W = 0.97921, p-value = 0.2321
```

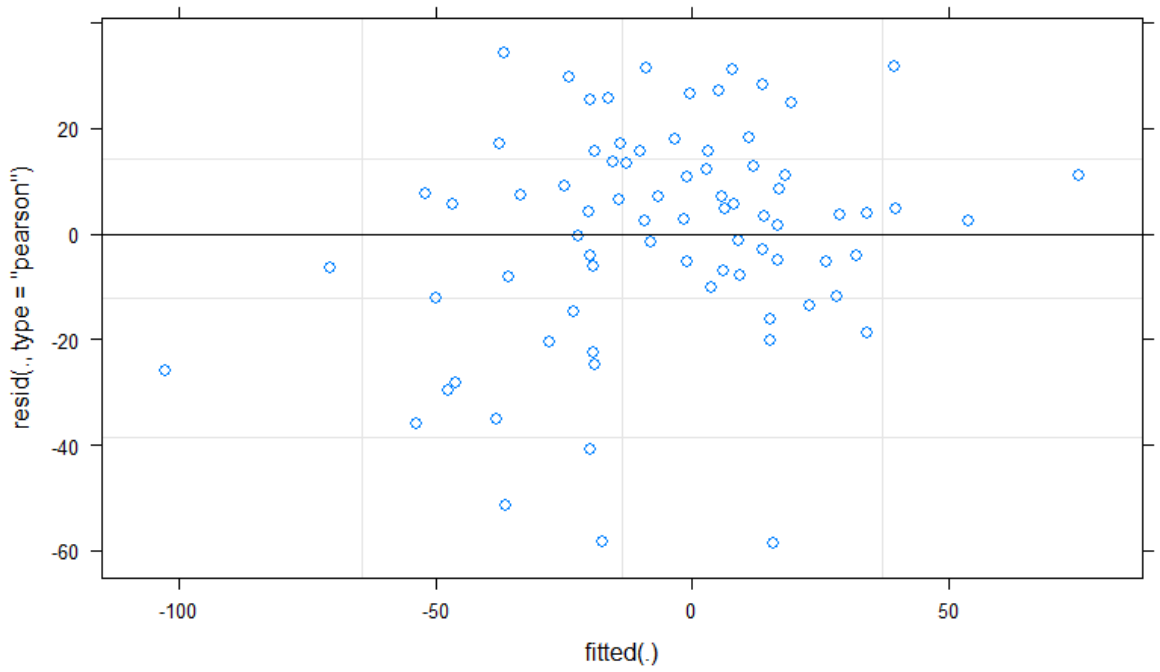
```
> plot(modswap, results="hide", fig.show='hide')
```



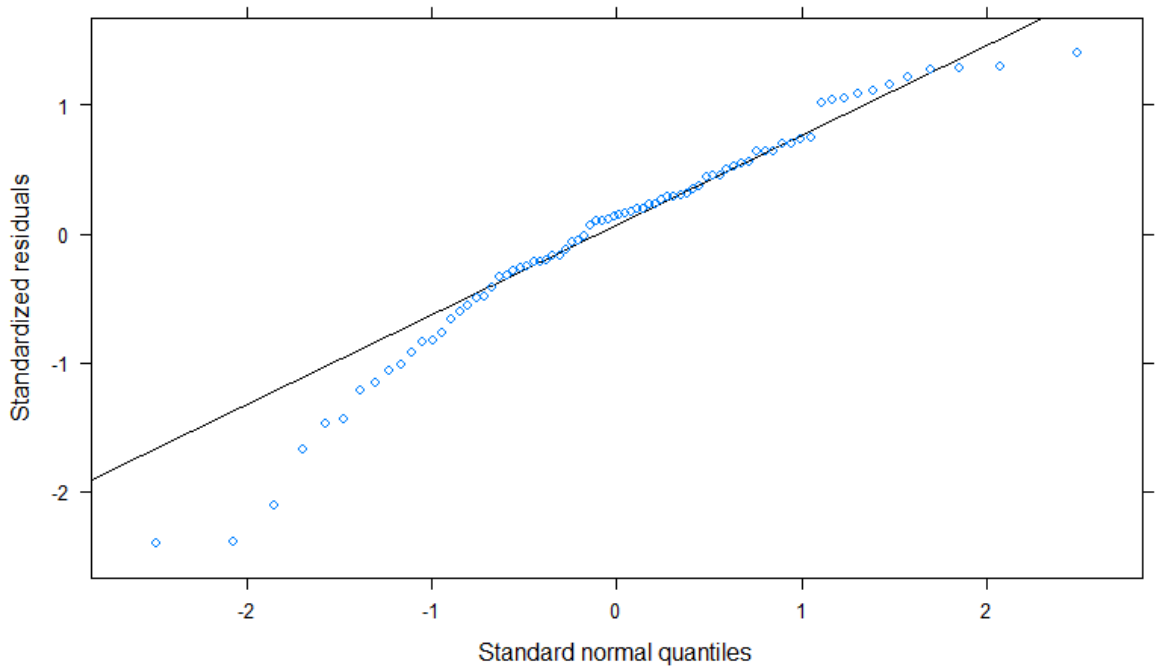
Now add Difference in control speeds as a fixed effect

```
> modswapB<-lmer(Difference.in.rivals.SF ~ as.factor(YEAR)
+SOCIAL.STATUS.SF + Difference.own.speeds  + (1|pair) +(1|WEEK)
+(1|Male.ID.sperm) +(1|Rivals.ID),data=SEMINALFLUIDSWAP)
> summary(modswapB)

## Linear mixed model fit by REML
## t-tests use  Satterthwaite approximations to degrees of freedom
##  ['lmerMod']
## Formula: Difference.in.rivals.SF ~ as.factor(YEAR) + SOCIAL.STATUS.SF +
## Difference.own.speeds +
##    (1 | pair) + (1 | WEEK) + (1 | Male.ID.sperm) + (1 | Rivals.ID)
##   Data: SEMINALFLUIDSWAP
##
## REML criterion at convergence: 720.7
##
## Scaled residuals:
##    Min      1Q  Median      3Q     Max
## -2.4029 -0.3984  0.1413  0.5410  1.4050
##
## Random effects:
## Groups        Name        Variance Std.Dev.
## Rivals.ID     (Intercept) 192.62   13.879
## Male.ID.sperm (Intercept)  60.82    7.799
## pair          (Intercept)   0.00    0.000
```

```
## WEEK          (Intercept)   0.00    0.000
## Residual                  595.21   24.397
## Number of obs: 78, groups:  Rivals.ID, 42; Male.ID.sperm, 42; pair, 39;
## WEEK, 5
##
## Fixed effects:
##                       Estimate Std. Error    df   t value  Pr(>|t|)
## (Intercept)           -12.474      7.909    34.640  1.577    0.124
## as.factor(YEAR)2014     2.11001    9.06235  28.570   0.233    0.818
## as.factor(YEAR)2015     5.26272    9.73634  26.650   0.541    0.593
## SS.SFSubdominant        8.65605    7.39091  64.100   1.171    0.246
## Difference.own.speeds   0.72143    0.09715  69.020   7.426 2.24e-10 ***
## ---
## Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
##
## Correlation of Fixed Effects:
##             (Intr) a.(YEAR)2014 a.(YEAR)2015 SS.SFS
## a.(YEAR)2014 -0.681
## a.(YEAR)2015 -0.635  0.554
## SS.SFSbdmnn  -0.468 -0.002        0.000
## Dffrnc.wn.s   0.223 -0.001       -0.001       -0.475
> confint(modswapB, level=0.95, method="Wald",oldNames=F) #generate 95%CI
using Wald method
##                                    2.5 %     97.5 %
## (Intercept)                   -27.9773639  3.0292051
## as.factor(YEAR)2014           -15.6518711 19.8718874
## as.factor(YEAR)2015           -13.8201595 24.3456017
## SS.SFSubdominant               -5.8298735 23.1419734
## Difference.own.speeds           0.5310128  0.9118528

> plot(modswapB, results="hide", fig.show='hide')
```

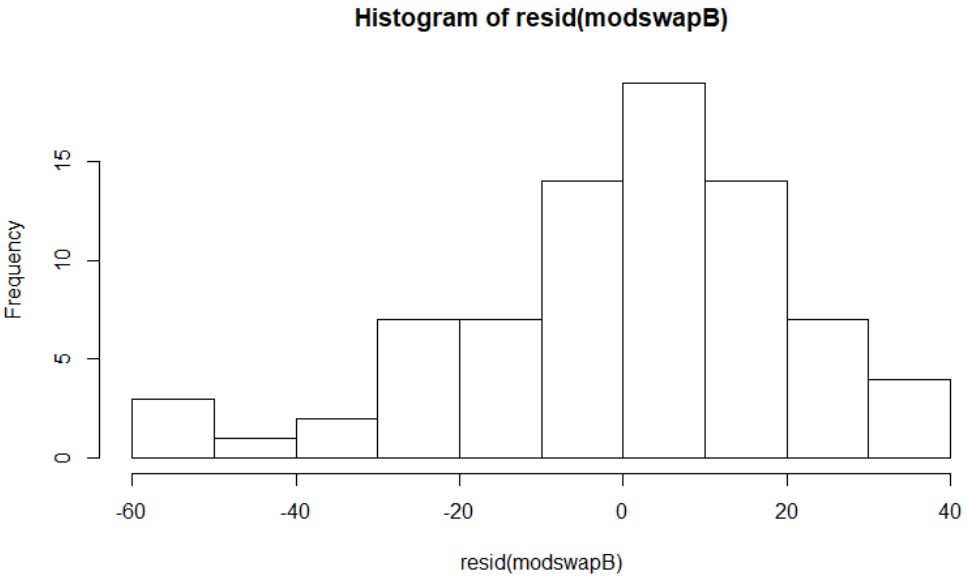


```
> qqmath(modswapB)
```




```
 > shapiro.test(resid(modswapB))
##
##    Shapiro-Wilk normality test

## data:  resid(modswapB)
## W = 0.95397, p-value = 0.006723
```

```
> hist(resid(modswapB))
```

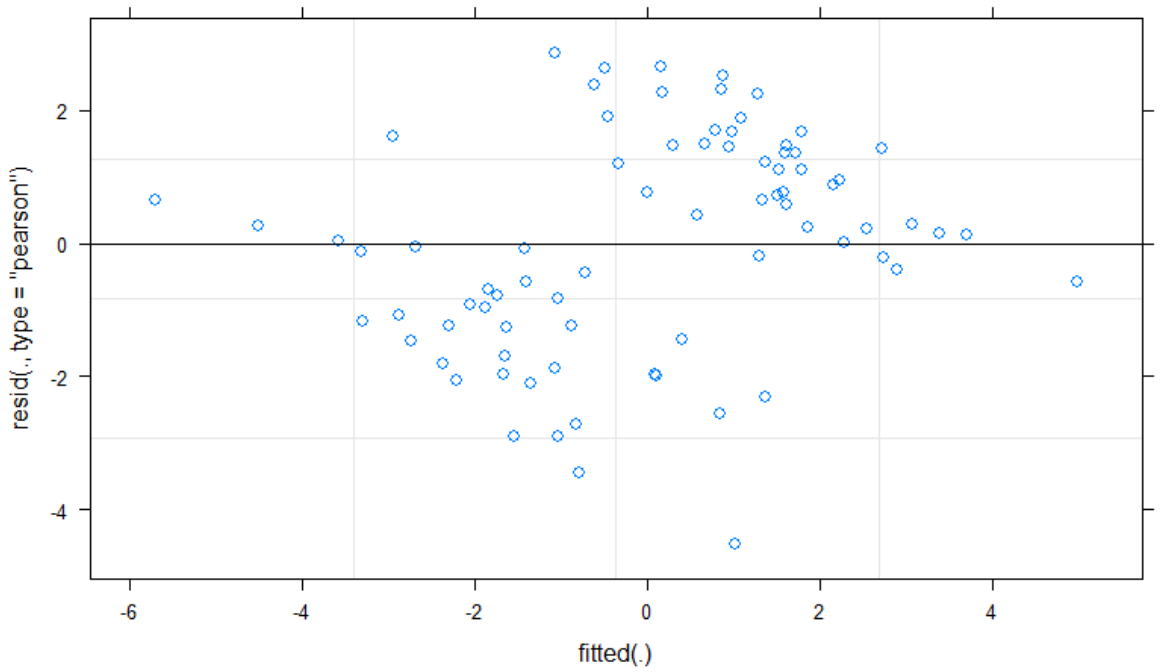



```
Has violated assumption of normality, will use transformation
```

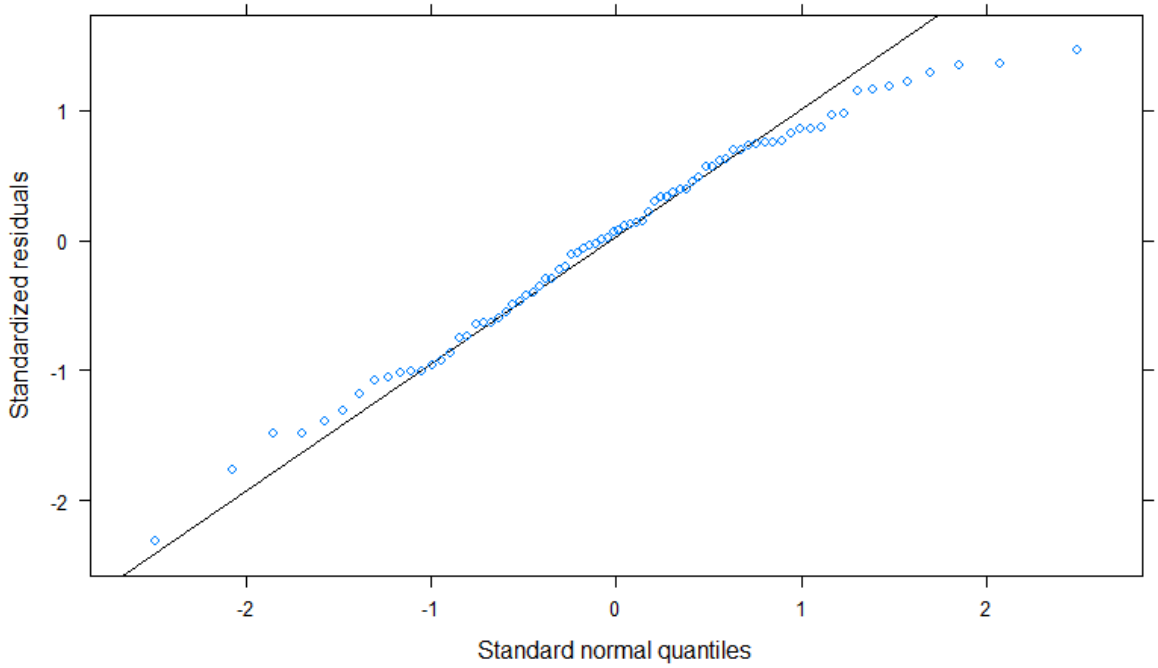
```
> Math.cbrt <- function(x) {
+    sign(x) * abs(x)^(1/3)} #Create function for cube root
transformation, this allows transformation of data to correct skew when
including negative values, better option than applying a constant and
then log transforming.


> modswapC<-lmer(Math.cbrt(Difference.in.rivals.SF) ~ as.factor(YEAR) +
SOCIAL.STATUS.SF + Difference.own.speeds  + (1|pair)
+(1|WEEK)+(1|Male.ID.sperm)+(1|Rivals.ID),data=SEMINALFLUIDSWAP)
> summary(modswapC)
## Linear mixed model fit by REML
## t-tests use  Satterthwaite approximations to degrees of freedom
## ['lmerMod']
## Formula: Math.cbrt(Difference.in.rivals.SF) ~ as.factor(YEAR) +
## SOCIAL.STATUS.SF +  Difference.own.speeds + (1 | pair) + (1 | WEEK) +
## (1 | Male.ID.sperm) +  (1 | Rivals.ID)
##  Data: SEMINALFLUIDSWAP

## REML criterion at convergence: 352.3
##
## Scaled residuals:
##     Min       1Q   Median       3Q      Max
## -2.31262 -0.62724  0.07258  0.69256  1.46382
##
## Random effects:
## Groups        Name        Variance Std.Dev.
## Rivals.ID     (Intercept) 0.7891   0.8883
## Male.ID.sperm (Intercept) 0.8081   0.8989
## pair          (Intercept) 0.0000   0.0000
## WEEK          (Intercept) 0.0000   0.0000
## Residual                  3.8187   1.9541
## Number of obs: 78, groups:  Rivals.ID, 42; Male.ID.sperm, 42; pair,
## 39; WEEK, 5
##
## Fixed effects:
##                      Estimate  Std. Error  df   t value  Pr(>|t|)
## (Intercept)           -0.643713  0.632289 34.1100  -1.018  0.316
## as.factor(YEAR)2014    0.282424  0.723268 27.9300  0.390   0.699
## as.factor(YEAR)2015    0.884680  0.776886 26.0100  1.139   0.265
## SS.SFSubdominant       0.436635  0.594831 67.6900  0.734   0.465
## Difference.own.speeds  0.050620  0.007802 69.1700   6.488  1.1e-08 ***
## ---
## Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
##
## Correlation of Fixed Effects:
##             (Intr) a.(YEAR)2014 a.(YEAR)2015 SS.SFS
## a.(YEAR)2014 -0.681
## a.(YEAR)2015 -0.634  0.554
## SS.SFSbdmnn  -0.470  0.000        0.000
## Dffrnc.wn.s   0.223  0.000        0.000       -0.475
```

```
> confint(modswapC, level=0.95, method="Wald",oldNames=F) #generate
95%CI using Wald method
##                                    2.5 %    97.5 %
## (Intercept)                   -1.88297629 0.5955510
## as.factor(YEAR)2014           -1.13515432 1.7000027
## as.factor(YEAR)2015           -0.63798882 2.4073494
## SS.SFSubdominant              -0.72921148 1.6024819
## Difference.own.speeds          0.03532906 0.0659114
```

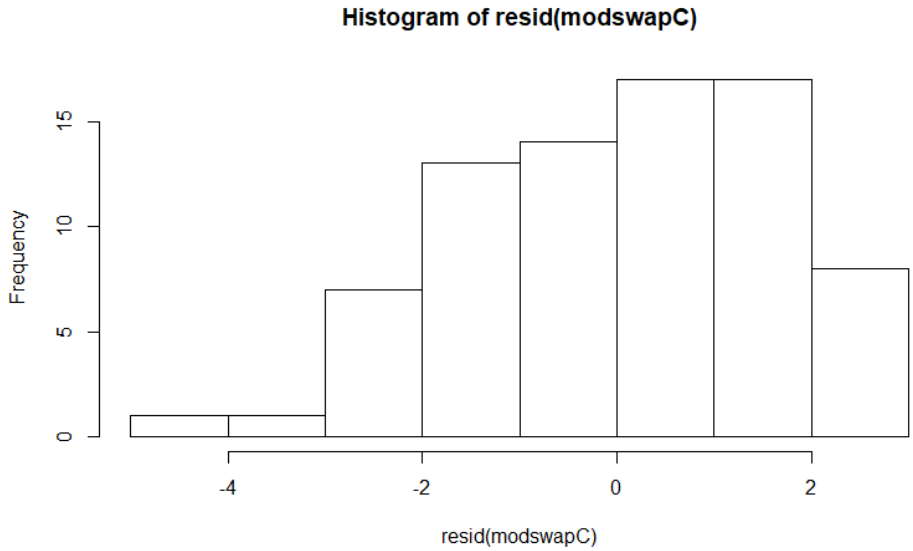
```
> plot(modswapC, results="hide", fig.show='hide')
```



```
> qqmath(modswapC)
```




```
> shapiro.test(resid(modswapC))
##
##    Shapiro-Wilk normality test
##
## data:  resid(modswapC)
## W = 0.97744, p-value = 0.181
## > hist(resid(modswapC))
```



Transformation appears to have pulled residuals towards normality, now passes shapiro test.

In-vitro fertilisation trials

We used both Fishers exact and G tests to determine if the paternity shares observed across replicate fertility trials were repeatable.

```
Replicates<-
read.table(file="FERT_TRIAL_REP_TEST.csv",header=T,row.names=NULL,sep=",")
#load Data sheet
#Fisher exact and Likelihood ratio (G) tests
pval <- NULL
Gpval <- NULL
count1 <- 0
count2 <- 0
count3 <- 0
count4 <- 0
a <- 1
b <- 2
f1 <- NULL
mat1 <- NULL
for (i in 1:41) {
  mat1 <- Replicates[a:b,5:6]
  #print(mat1) #remove comment if you want to show each 2x2 matrix
  c1 <- fisher.test(mat1)
  c2 <- likelihood.test(mat1) #Pete Hurd's Likelihood Ratio (G-test) for
contingency tables
  pval[i] <- c1$p.value #P from the Fisher tests
  Gpval[i] <- c2$p.value #P from the G-tests
  if(pval[i] <0.05) count1 <- count1+1
  if(pval[i] <0.0006) count2 <- count2+1
  if(Gpval[i] <0.05) count3 <- count3+1
  if(Gpval[i] <0.0006) count4 <- count4+1
  a=a+2
  b=b+2
}

> count1 #(pval[i] <0.05)
## [1] 2
> count2 #(pval[i] <0.0006)
## [1] 0
> count3 #(Gpval[i] <0.05)
## [1] 5
> count4 #(Gpval[i] <0.0006)
## [1] 1
#Adjusted p and G values using both holm and bonferroni methods
> p.adjust(pval, method="holm")
##[1] 1.0000000 1.0000000 1.0000000 1.0000000 1.0000000 1.0000000
##1.0000000 1.0000000 1.0000000 1.0000000
##[11] 1.0000000 1.0000000 1.0000000 1.0000000 1.0000000 1.0000000
##1.0000000 1.0000000 0.1598219 1.0000000
##[21] 1.0000000 1.0000000 1.0000000 1.0000000 1.0000000 1.0000000
##1.0000000 1.0000000 1.0000000 1.0000000
##[31] 1.0000000 1.0000000 1.0000000 1.0000000 1.0000000 1.0000000
##1.0000000 1.0000000 1.0000000 1.0000000
##[41] 1.0000000
```

```
> p.adjust(pval, method="bonferroni")
##[1] 1.0000000 1.0000000 1.0000000 1.0000000 1.0000000 1.0000000
##1.0000000 1.0000000 1.0000000 1.0000000
##[11] 1.0000000 1.0000000 1.0000000 1.0000000 1.0000000 1.0000000
##1.0000000 1.0000000 0.1598219 1.0000000
##[21] 1.0000000 1.0000000 1.0000000 1.0000000 1.0000000 1.0000000
##1.0000000 1.0000000 1.0000000 1.0000000
##[31] 1.0000000 1.0000000 1.0000000 1.0000000 1.0000000 1.0000000
##1.0000000 1.0000000 1.0000000 1.0000000
##[41] 1.0000000
> p.adjust(Gpval, method="holm")
##[1] 1.00000000 1.00000000 1.00000000 1.00000000 1.00000000 1.00000000
##1.00000000 1.00000000 1.00000000
##[10] 1.00000000 1.00000000 1.00000000 1.00000000 1.00000000 1.00000000
##1.00000000 1.00000000 1.00000000
##[19] 0.01498229 1.00000000 1.00000000 1.00000000 1.00000000 1.00000000
##1.00000000 1.00000000 1.00000000
##[28] 1.00000000 1.00000000 1.00000000 1.00000000 1.00000000 0.24485998
##1.00000000 1.00000000 1.00000000
##[37] 1.00000000 1.00000000 1.00000000 1.00000000 1.00000000
> p.adjust(Gpval, method="bonferroni")
##[1] 1.00000000 1.00000000 1.00000000 1.00000000 1.00000000 1.00000000
##1.00000000 1.00000000 1.00000000
##[10] 1.00000000 1.00000000 1.00000000 1.00000000 1.00000000 1.00000000
##1.00000000 1.00000000 1.00000000
##[19] 0.01498229 1.00000000 1.00000000 1.00000000 1.00000000 1.00000000
##1.00000000 1.00000000 1.00000000
##[28] 1.00000000 1.00000000 1.00000000 1.00000000 1.00000000 0.25098148
##1.00000000 1.00000000 1.00000000
##[37] 1.00000000 1.00000000 1.00000000 1.00000000 1.00000000

Replicates for trial 19 were removed from further analysis as paternity
share differed significantly between replicates.
```

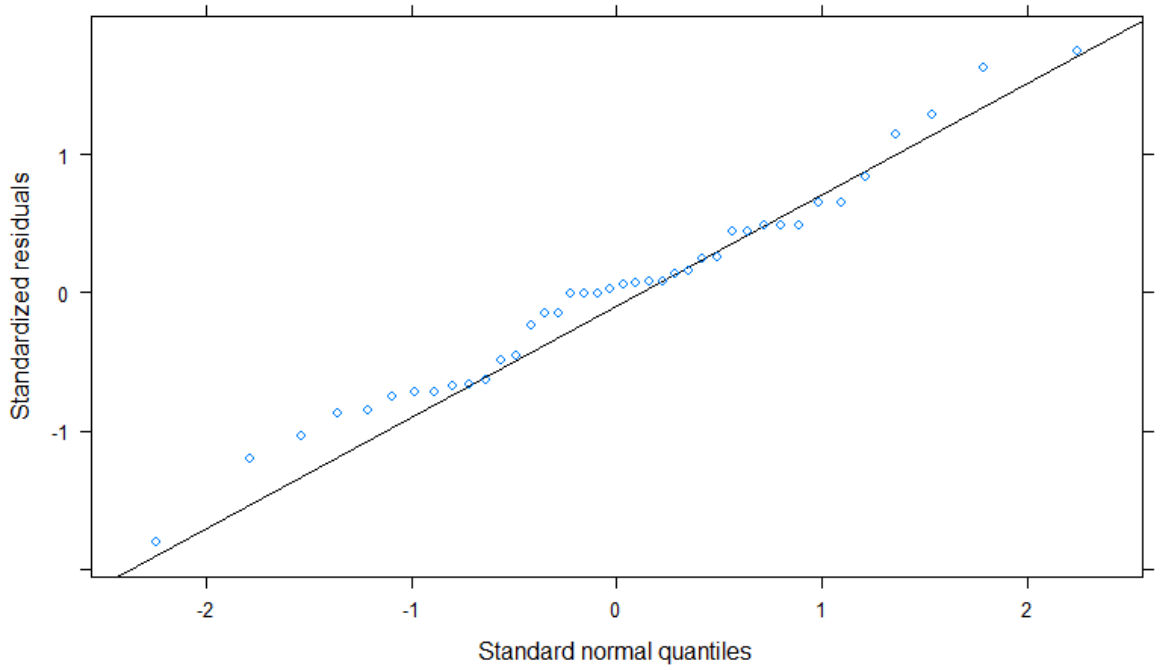
Relative sperm velocity as a predictor of fertilisation success (in both seminal fluid treatments)

In-vitro fertilisation trials using unmanipulated milt

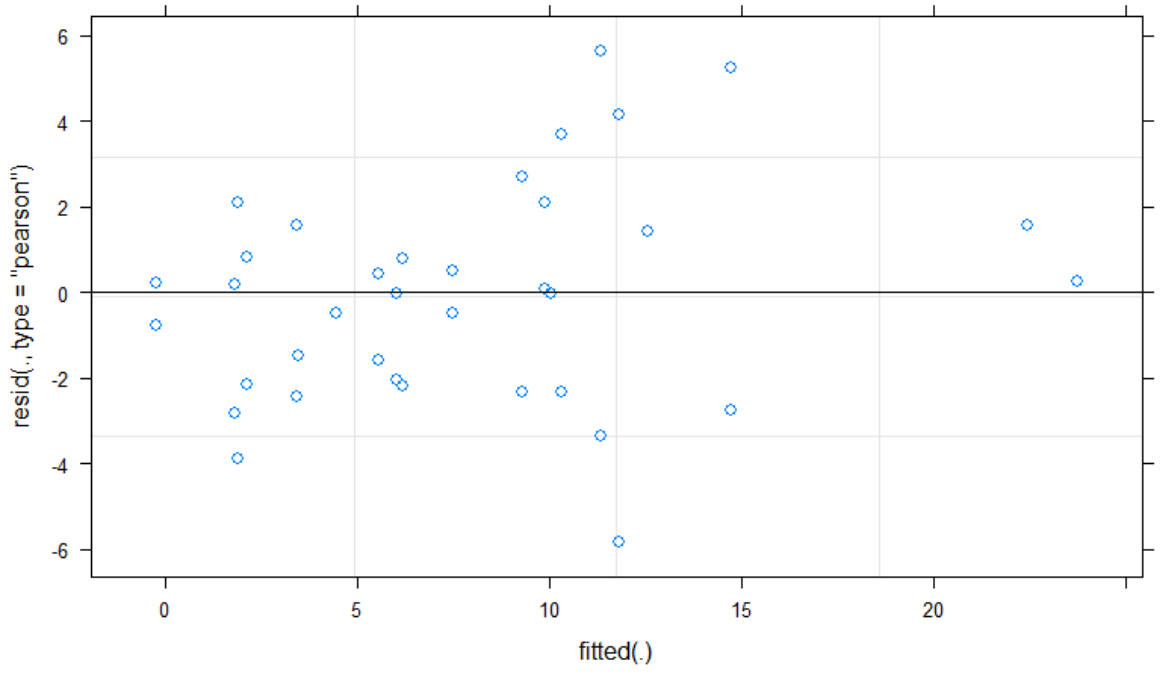
```
>TRIALSM2<-
read.table(file="FERT_REL_VAP_MILT.csv",header=T,row.names=NULL,sep=",")#l
oad Data sheet

> TRIALSNEW<-TRIALSM2[c(-38,-39),] #remove trial 19 from the data that
failed the replicability tests (see above)

> #does relative sperm velocity (measured in OF) predict fert success?

## Linear mixed model fit by REML
## t-tests use  Satterthwaite approximations to degrees of freedom
## ['lmerMod']
## Formula: DiffFERTmaleA ~ YEAR + DiffVAPof + (1 | WEEK) + (1 | maleA) +
##    (1 | maleB) + (1 | Female) + (1 | TRYAD)
```

```
##   Data: TRIALSNEW
##
##REML criterion at convergence: 240.5
##
## Scaled residuals:
##     Min       1Q   Median       3Q      Max
## -1.80443 -0.63631  0.04371  0.45178  1.74778
##
## Random effects:
##  Groups   Name        Variance  Std.Dev.
##  TRYAD    (Intercept) 1.054e+01 3.247e+00
##  Female   (Intercept) 1.836e-07 4.285e-04
##  maleA    (Intercept) 2.077e+01 4.558e+00
##  maleB    (Intercept) 3.743e-07 6.118e-04
##  WEEK     (Intercept) 2.130e-09 4.615e-05
##  Residual             1.044e+01 3.231e+00
## Number of obs: 40, groups:  TRYAD, 21; Female, 17; maleA, 17; maleB, 15
## ; WEEK, 4

## Fixed effects:
##              Estimate Std. Error         df t value Pr(>|t|)
## (Intercept) -1.488e+03  5.774e+03  1.998e+01  -0.258  0.79926
## YEAR         7.419e-01  2.866e+00  1.998e+01   0.259  0.79842
## diffVAPof    1.443e-01  4.313e-02  2.542e+01   3.345  0.00256 **
## ---
## Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

## Correlation of Fixed Effects:
##          (Intr) YEAR
## YEAR      -1.000
## diffVAPof -0.057  0.057

> confint(modA.2, level=0.95, method="Wald",oldNames=F) #generate 95%CI
using Wald method
##                             2.5 %        97.5 %
## (Intercept)           -1.280511e+04 9828.925960
## YEAR                  -4.875879e+00    6.359599
## diffVAPof              5.975185e-02    0.228811
```

```
> qqmath(modA.2)
```



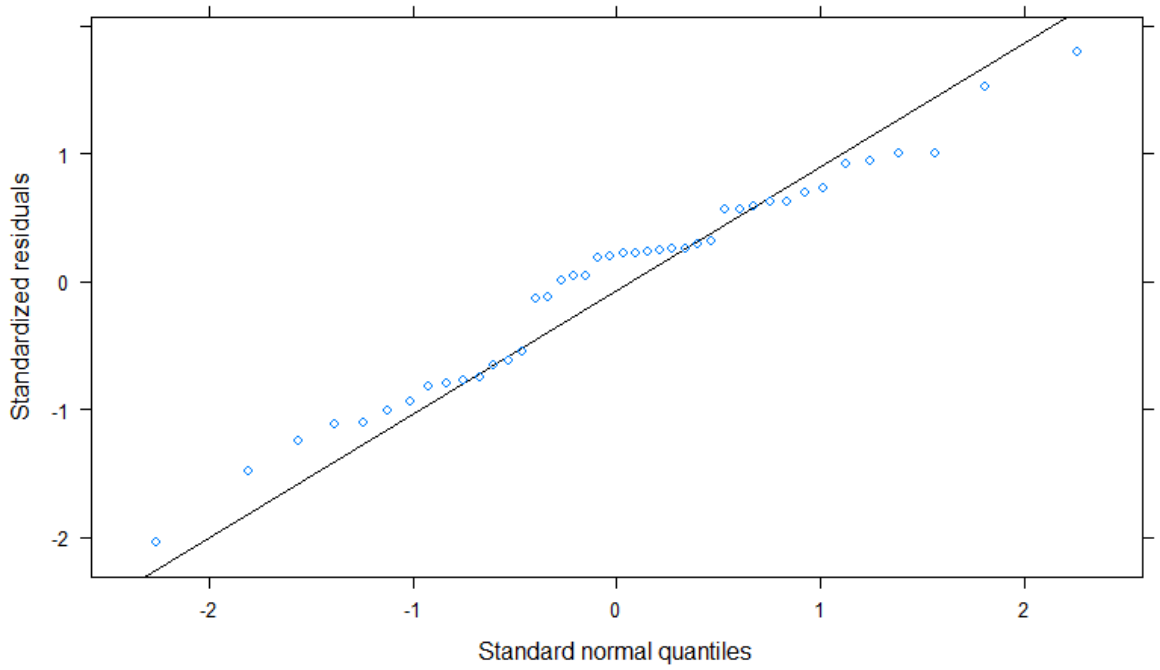

```
> shapiro.test(resid(modA.2))

## Shapiro-Wilk normality test
##
## data:  resid(modA.2)
## W = 0.98044, p-value = 0.7059

> plot(modA.2, results="hide", fig.show='hide')
```



In-vitro fertilisation trials using manipulated milt (seminal fluid and sperm swapped between focal and rival male in each trial).

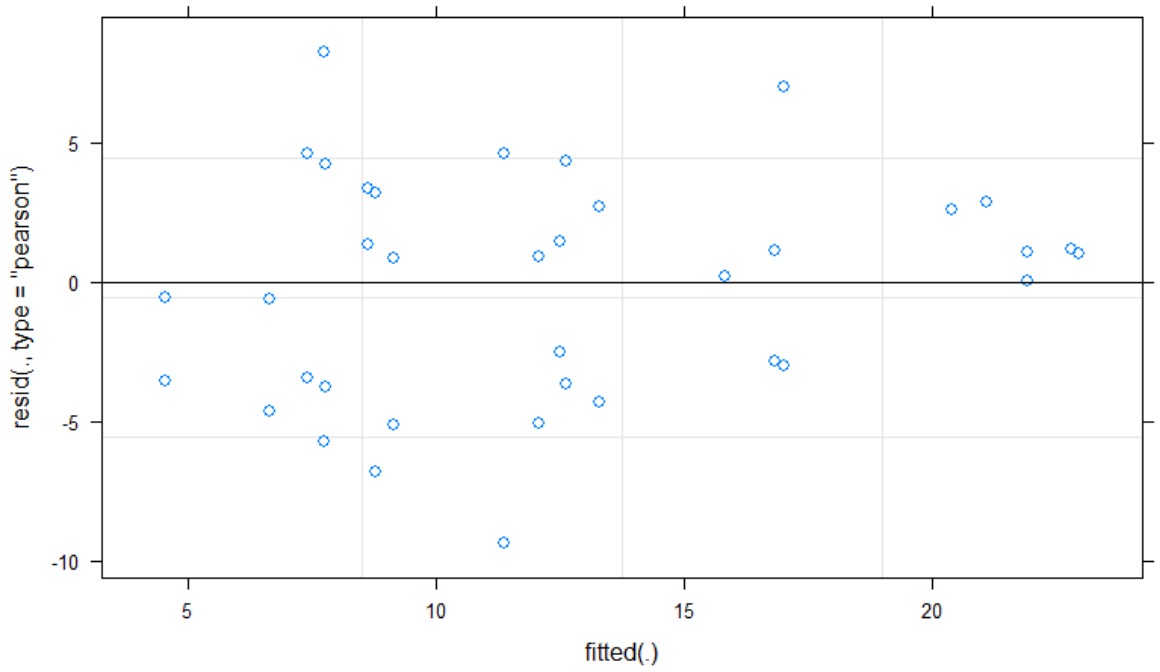
```
>TRIALSSWAP<-
read.table(file="FERT_REL_VAP_SWAP.csv",header=T,row.names=NULL,sep=",")
#load Data sheet

> #does relative sperm velocity (measured in OF) predict fert success?

## Linear mixed model fit by REML
## t-tests use  Satterthwaite approximations to degrees of freedom
## ['lmerMod']
## Formula: DiffFERTmaleA ~ YEAR + DiffVAPof + (1 | WEEK) + (1 | maleA) +
##    (1 | maleB) + (1 | Female) + (1 | TRYAD)
##   Data: TRIALSSWAP
##
## REML criterion at convergence: 265.8
##
## Scaled residuals:
##    Min      1Q  Median      3Q     Max
## -2.0423 -0.7212  0.2128  0.5847  1.8003
##
## Random effects:
##  Groups   Name        Variance  Std.Dev.
##  TRYAD    (Intercept) 1.002e-07 0.0003165
##  Female   (Intercept) 2.889e+01 5.3752530
##  maleB    (Intercept) 2.991e-06 0.0017296
##  maleA    (Intercept) 0.000e+00 0.0000000
##  WEEK     (Intercept) 0.000e+00 0.0000000
##  Residual             2.108e+01 4.5908229
## Number of obs: 42, groups:  TRYAD, 21; Female, 16; maleB, 16; maleA,
## 16; WEEK, 4
##
## Fixed effects:
##              Estimate Std. Error       df     t value   Pr(>|t|)
## (Intercept) 3722.73905 6293.48426   19.22000   0.592  0.56106
## YEAR          -1.84273    3.12406   19.22000  -0.590  0.56216
## diffVAPof      0.13489    0.04216   36.55000   3.200  0.00284 **
## ---
## Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
##
## Correlation of Fixed Effects:
##          (Intr) YEAR
## YEAR      -1.000
## diffVAPof  0.157 -0.157
> confint(modB.2, level=0.95, method="Wald",oldNames=F) #generate 95%CI
using Wald method
##                            2.5 %         97.5 %
## (Intercept)          -8.612263e+03 1.605774e+04
## YEAR                 -7.965773e+00 4.280319e+00
## diffVAPof             5.226232e-02 2.175134e-01
```

```
> qqmath(modB.2)
```




```
> shapiro.test(resid(modB.2))

## Shapiro-Wilk normality test
##
## data:  resid(modB.2)
## W = 0.97372, p-value = 0.4366
```

```
> plot(modB.2, results="hide", fig.show='hide')
```



Comparing the proportion of offspring sired by males of different social status (in both seminal fluid treatments)

unmanipulated milt trials:

```
TRIALS<-
read.table(file="FERT_SS_MILT.csv",header=T,row.names=NULL,sep=",") #load
Data sheet
TNEW <-TRIALS[c(-75,-76,-77,-78),] #remove trial 20 from the data that
failed the replicability tests (see above)
> modX<-glmer(PFERT~SS+(1|MaleID)+(1|Female)+(1|TRYAD)+(1|WEEK),
 family="binomial", weights=TOTAL ,data=TNEW)
> summary(modX)
## Generalized linear mixed model fit by maximum likelihood (Laplace
## Approximation) ['glmerMod']
## Family: binomial  ( logit )
## Formula: PFERT ~ SS + (1 | MaleID) + (1 | Female) + (1 | TRYAD) +
## (1 | WEEK)
## Data: TNEW
## Weights: TOTAL
##
##    AIC      BIC   logLik deviance df.resid
##   467.8    482.1   -227.9    455.8       74
##
## Scaled residuals:
##    Min       1Q   Median       3Q      Max
## -2.06803 -0.43276  0.00992  0.45587  1.69527
##
## Random effects:
## Groups Name        Variance  Std.Dev.
## MaleID (Intercept) 2.156e+00 1.468e+00
## TRYAD  (Intercept) 5.400e-01 7.348e-01
## Female (Intercept) 1.311e-09 3.621e-05
## WEEK   (Intercept) 4.502e-09 6.710e-05
## Number of obs: 80, groups:  MaleID, 24; TRYAD, 21; Female, 17; WEEK, 4
##
## Fixed effects:
##             Estimate Std. Error z value Pr(>|z|)
## (Intercept)  -0.3789     0.3655  -1.037      0.3
## SSS           1.1052     0.2400   4.604 4.14e-06 ***
## ---
## Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
##
## Correlation of Fixed Effects:
##   (Intr)
## SSS -0.320
> confint(modX, level=0.95, method="Wald",oldNames=F)
##                            2.5 %    97.5 %
## (Intercept)           -1.0952987 0.3375164
## SSS                    0.6347259 1.5756610
> overdisp.glmer(modX)# check for over/under-dispersion
## Residual deviance: 63.209 on 74 degrees of freedom (ratio: 0.854)
```

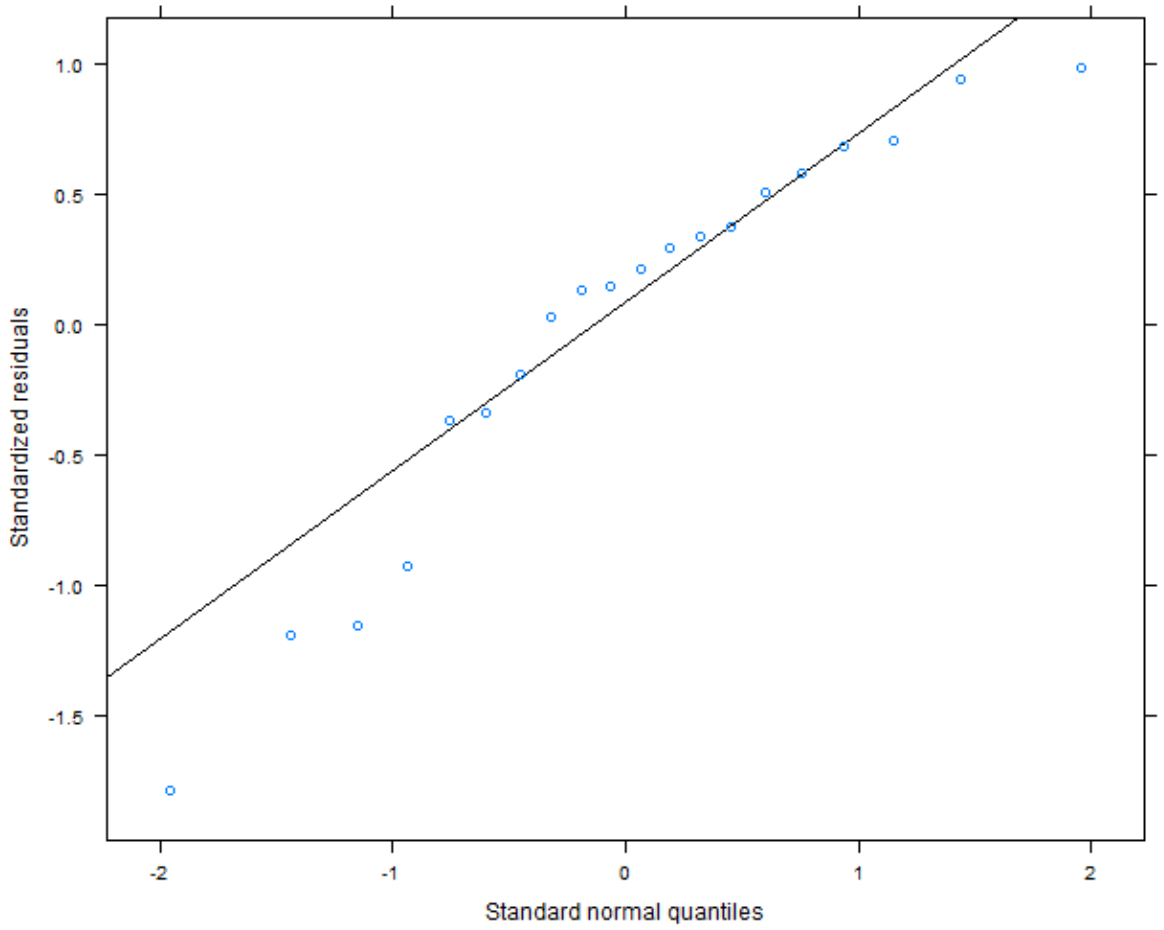
Seminal fluid swapped trials:

```
>TRIALS2<-
read.table(file="FERT_SS_SWAP.csv",header=T,row.names=NULL,sep=",")
#load Data sheet
> #Swapped seminal fluid trials, social status of seminal fluid in
 which sperm were incubated as fixed predictor
> modX2<-glmer(PFERT~SS.SF+(1|maleID)+(1|Female)+(1|TRYAD)+(1|WEEK),
family="binomial", weights=TOTAL ,data=TRIALS2)
> summary(modX2)
## Generalized linear mixed model fit by maximum likelihood (Laplace
## Approximation) ['glmerMod']
## Family: binomial  ( logit )
## Formula: PFERT ~ SS.SF + (1 | maleID) + (1 | Female) + (1 | TRYAD) +
## (1 | WEEK)
##  Data: TRIALS2
## Weights: TOTAL
##
##    AIC      BIC   logLik deviance df.resid
##  537.1    551.7   -262.5    525.1       78
##
## Scaled residuals:
##     Min       1Q   Median       3Q      Max
## -2.41280 -0.55202 -0.02723  0.58103  2.36920
##
## Random effects:
## Groups Name        Variance Std.Dev.
## maleID (Intercept) 25.29    5.029
## TRYAD  (Intercept) 13.87    3.725
## Female (Intercept)  0.00    0.000
## WEEK   (Intercept)  0.00    0.000
## Number of obs: 84, groups:  maleID, 24; TRYAD, 21; Female, 16; WEEK, 4
##
## Fixed effects:
##           Estimate Std. Error z value Pr(>|z|)
## (Intercept)   -3.241      1.381  -2.347   0.0189 *
## SS.SFS         6.225      0.766   8.126 4.44e-16 ***
## ---
## Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
##
## Correlation of Fixed Effects:
##      (Intr)
## SS.SFS -0.283
> confint(modX2, level=0.95, method="Wald",oldNames=F)
##                           2.5 %     97.5 %
## (Intercept)           -5.947186 -0.5339593
## SS.SFS                 4.723309  7.7261285
> overdisp.glmer(modX2)# check for over/under-dispersion
## Residual deviance: 86.337 on 78 degrees of freedom (ratio: 1.107)
```

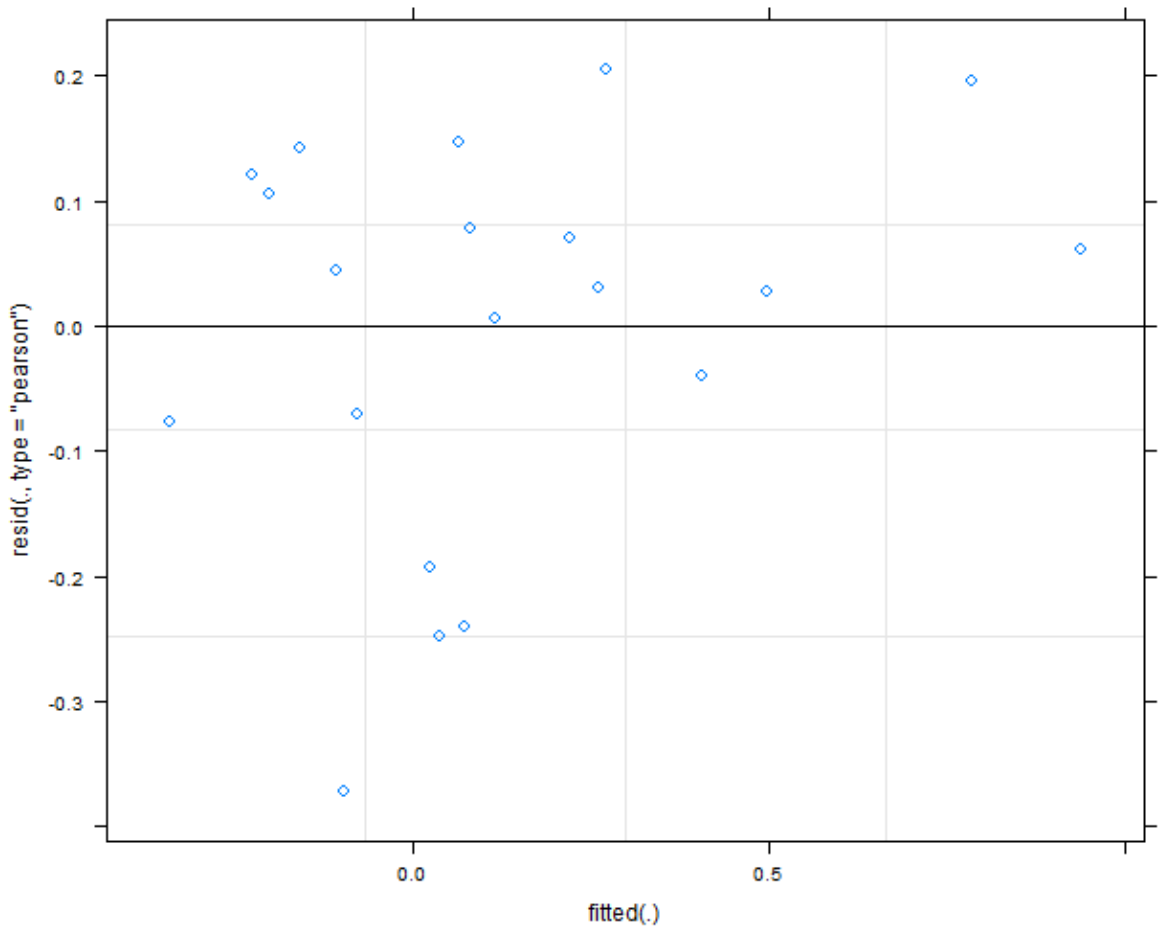
Testing relationship between the change in number of eggs fertilised and the change in relative sperm velocity across seminal fluid treatments within the same male-male-female combinations.

```
>TREATEFF<-
read.table(file="DIFF_ACROSS_TREATMENTS.csv",header=T,row.names=NULL,sep="
,")#load Data sheet
> TRIALSNEW2<-TREATEFF[c(-19),] #remove trial 20 from the data that failed
the replicability tests (see above)
> modC<-lmer(DIFFPFERT ~ DIFFVAPof + YEAR+
(1|maleA)+(1|maleB)+(1|WEEK),data=TRIALSNEW2)
> summary(modC)
## Linear mixed model fit by REML t-tests use Satterthwaite
## approximations to degrees of freedom [lmerMod]
## Formula:
## DIFFPFERT ~ DIFFVAPof + YEAR + (1 | maleA) + (1 | maleB) +
## (1 | WEEK)
## Data: TRIALSNEW2
##
## REML criterion at convergence: 18.3
##
## Scaled residuals:
##   Min      1Q    Median    3Q     Max
## -1.7865 -0.3430  0.1815  0.5275  0.9868
##
## Random effects:
## Groups   Name        Variance Std.Dev.
## maleA    (Intercept) 0.022522 0.15007
## maleB    (Intercept) 0.004506 0.06713
## WEEK     (Intercept) 0.000000 0.00000
## Residual             0.043366 0.20824
## Number of obs: 20, groups:  maleA, 17; maleB, 15; WEEK, 4
##
## Fixed effects:
##              Estimate   Std. Error     df     t value Pr(>|t|)
## (Intercept) -56.398420 252.018591  14.297000  -0.224 0.826090
## DIFFVAPof     0.005577   0.001034  12.997000   5.395 0.000122 ***
## YEAR          0.028045   0.125102  14.297000   0.224 0.825788
## ---
## Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
##
## Correlation of Fixed Effects:
##           (Intr) DIFFVA
## DIFFVAPof -0.045
## YEAR      -1.000  0.045

> confint(modC, level=0.95, method="Wald",oldNames=F) #generate 95%CI
using Wald method
##                              2.5 %           97.5 %
## (Intercept)          -5.503458e+02     4.375489e+02
## DIFFVAPof             3.550875e-03     7.603404e-03
## YEAR                 -2.171491e-01     2.732401e-01
```

```
> qqmath(modC)
```




```
> shapiro.test(resid(modC))

##    Shapiro-Wilk normality test
##
## data:  resid(modC)
## W = 0.91891, p-value = 0.09442

> plot(modC, results="hide", fig.show='hide')
```


0.2
0.1
0.0
-0.1
-0.2
-0.3
resid(., type = "pearson")
0.0
0.5
fitted(.)

References

[1] R Core Team (2016) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
URL: https://www.R-project.org/

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[3] Sarkar D. (2008) Lattice: Multivariate Data Visualization with R. Springer, New York. ISBN 978-0-387-75968-5

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APPENDIX C:

CHAPTER THREE: STATISTICAL ANALYSIS AND R CODE

STATISTICAL ANALYSIS AND R CODE

CONTENTS **PAGE**

```
library(lme4)
library(lmerTest)
library(lattice)
library(ggplot2)
library(MASS)
library(car)
library(LMERConvenienceFunctions)
library(multcomp)
library(lsmeans)
library(readxl)
library(readr)
```

Effect of centrifuging sperm on VAP in both tactics

```
TreatmentTest1 <- read_excel("Hooknose v sneaker SWOP experiment 2016.xlsx",
    sheet = "TreatmentTest1") #Load data

mod4<-lmer(VAP ~ Treatment * Tactic + (1|MaleID), data=TreatmentTest1)
summary(mod4)
```

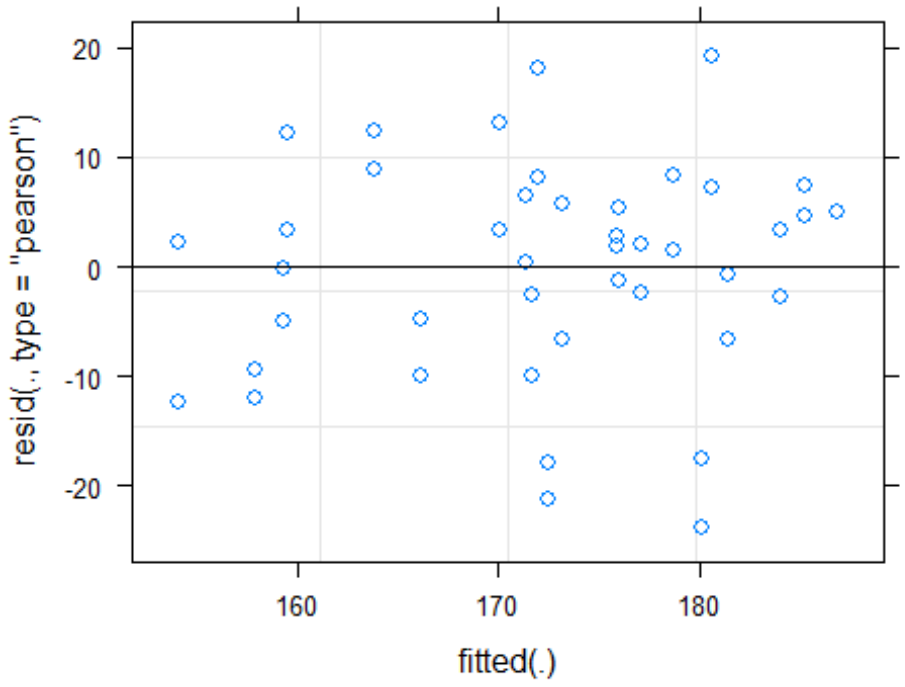
```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: VAP ~ Treatment * Tactic + (1 | MaleID)
##    Data: TreatmentTest1
##
## REML criterion at convergence: 317.9
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -2.2351 -0.5111  0.1772  0.5487  1.8035
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept)  22.25    4.717
##  Residual             114.40   10.696
## Number of obs: 44, groups:  MaleID, 11
##
## Fixed effects:
##                                Estimate Std. Error      df t value
## (Intercept)                     158.930      3.986  20.123  39.869
## TreatmentMilt                    12.100      4.783  31.000   2.530
## TacticPrecocious                 15.953      5.397  20.123   2.956
## TreatmentMilt:TacticPrecocious   -3.925      6.477  31.000  -0.606
##                                Pr(>|t|)
## (Intercept)                     < 2e-16 ***
## TreatmentMilt                   0.01672 *
## TacticPrecocious                0.00778 **
## TreatmentMilt:TacticPrecocious  0.54892
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
##
## Correlation of Fixed Effects:
##             (Intr) TrtmnM TctcPr
## TreatmntMlt -0.600
## TacticPrccs -0.739  0.443
## TrtmntMl:TP  0.443 -0.739 -0.600
```

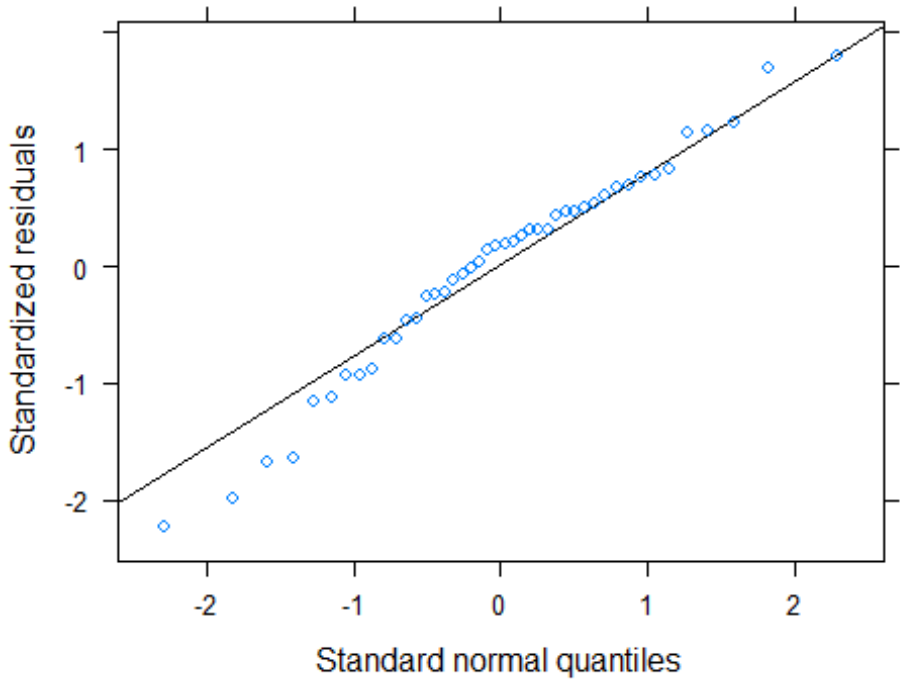
```
confint(mod4, level=0.95, method="Wald",oldNames=F) #generate 95%CI using Wal
d method
```

```
##                                       2.5 %     97.5 %
## sd_(Intercept)|MaleID                    NA         NA
## sigma                                    NA         NA
## (Intercept)                      151.117018 166.742982
## TreatmentMilt                      2.724712  21.475288
## TacticPrecocious                   5.374506  26.532161
## TreatmentMilt:TacticPrecocious   -16.619199   8.769199
```

```
#Model diagnostics
plot(mod4, results="hide", fig.show='hide') #plot residuals vs fitted values
to check for unequal variance
```



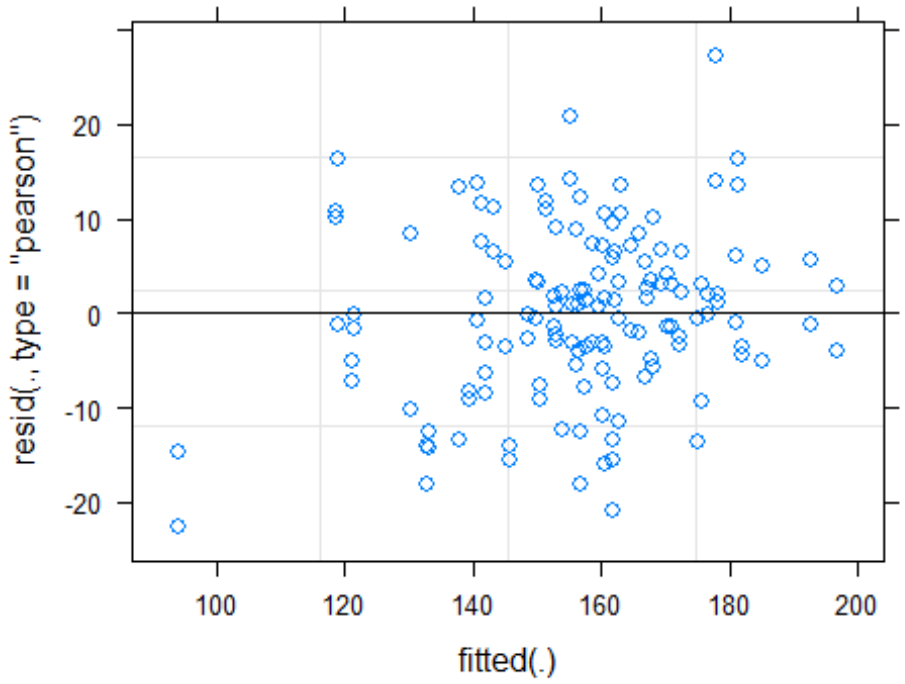

```
qqmath(mod4) #check normality assumption
```



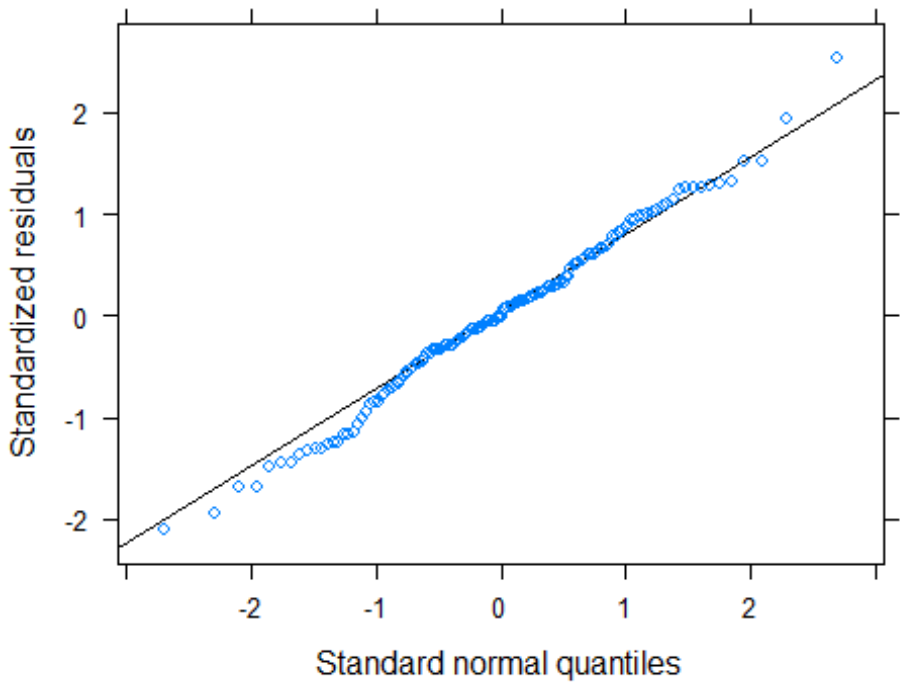

```
shapiro.test(resid(mod4))

##
##  Shapiro-Wilk normality test
##
## data:  resid(mod4)
## W = 0.97173, p-value = 0.348

rand(mod4)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID   1.27      1     0.3
```

Testing for tactic specific effects of seminal fluid on VAP

```
TreatmentTest2 <- read_excel("Hooknose v sneaker SWOP experiment 2016.xlsx",
    sheet = "TreatmentTest3") #Load data

mod3.2<-lmer(VAP ~  Sperm.Tactic * Treatment + (1|MaleID)+(1|PAIR),data=Treat
mentTest2)
summary(mod3.2)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: VAP ~ Sperm.Tactic * Treatment + (1 | MaleID) + (1 | PAIR)
##    Data: TreatmentTest2
##
## REML criterion at convergence: 1119.7
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -2.11207 -0.46362 -0.01931  0.56401  2.54344
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  PAIR     (Intercept) 170.8    13.07
##  MaleID   (Intercept) 186.1    13.64
##  Residual             115.2    10.73
## Number of obs: 138, groups:  PAIR, 42; MaleID, 12
##
## Fixed effects:
##                                     Estimate Std. Error      df t value
## (Intercept)                          158.930      9.106  25.280  17.454
## Sperm.TacticPrecocious                17.703     12.243  26.210   1.446
## TreatmentSwitch                        7.797      7.315  36.930   1.066
## Sperm.TacticPrecocious:TrtmentSwitch -38.557      9.381  40.430  -4.110
##                                     Pr(>|t|)
## (Intercept)                         1.33e-15 ***
## Sperm.TacticPrecocious              0.160029
## TreatmentSwitch                     0.293421
## Sperm.TacticPrecocious:TrtmentSwitch 0.000188 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) Spr.TP TrtmnS
## Sprm.TctcPr -0.744
## TrtmntSwtch -0.686  0.506
## Sprm.TcP:TS  0.535 -0.730 -0.698

#Model diagnostics
plot(mod3.2, results="hide", fig.show='hide') #plot residuals vs fitted value
s to check for unequal variance
```




```
qqmath(mod3.2) #check normality assumption
```




```
shapiro.test(resid(mod3.2))
```

```
##
##  Shapiro-Wilk normality test
##
## data:  resid(mod3.2)
## W = 0.99302, p-value = 0.7356
```

```
confint(mod3.2, level=0.95, method="Wald",oldNames=F) #generate 95%CI using W
ald method
```

```
##                                             2.5 %    97.5 %
## sd_(Intercept)|PAIR                            NA        NA
## sd_(Intercept)|MaleID                          NA        NA
## sigma                                          NA        NA
## (Intercept)                           141.083439 176.77656
## Sperm.TacticPrecocious                 -6.292307  41.69775
## TreatmentSwitch                        -6.540812  22.13436
## Sperm.TacticPrecocious:TrtmentSwitch -56.943818 -20.17045
```

```
lsmeans(mod3.2, ~ Sperm.Tactic|Treatment, cov.reduce = FALSE)
```

```
## Treatment = Control:
##  Sperm.Tactic   lsmean       SE    df lower.CL upper.CL
##  Hooknose     158.9300 9.105556 25.28 140.1871 177.6729
##  Precocious   176.6327 8.183507 27.41 159.7878 193.4777
##
## Treatment = Switched:
##  Sperm.Tactic   lsmean       SE    df lower.CL upper.CL
##  Hooknose     166.7268 6.712232 10.27 152.9103 180.5432
##  Precocious   145.8724 5.955245 11.64 133.6141 158.1306
##
## Degrees-of-freedom method: satterthwaite
## Confidence level used: 0.95
```

```
summary(glht(mod3.2, lsm(pairwise ~Treatment|Sperm.Tactic)))#Post hoc test fo
r mod3.2
```

```
## Note: df set to 37
```

```
## $`Sperm.Tactic = Hooknose`
##
##   Simultaneous Tests for General Linear Hypotheses
##
## Fit: lme4::lmer(formula = VAP ~ Sperm.Tactic * Treatment + (1 | MaleID) +
##     (1 | PAIR), data = TreatmentTest2)
##
## Linear Hypotheses:
##                       Estimate Std. Error t value Pr(>|t|)
## Control - Swap == 0   -7.797      7.315  -1.066    0.293
## (Adjusted p values reported -- single-step method)
##
##
```

```
## $`Sperm.Tactic = Precocious`
##
##   Simultaneous Tests for General Linear Hypotheses
##
## Fit: lme4::lmer(formula = VAP ~ Sperm.Tactic * Treatment + (1 | MaleID) +
##     (1 | PAIR), data = TreatmentTest2)
##
## Linear Hypotheses:
##                      Estimate Std. Error t value Pr(>|t|)
## Control - Swap == 0    30.76       6.76    4.55 5.61e-05 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## (Adjusted p values reported -- single-step method)
```

Testing for quality driven effect of seminal fluid on VAP

```
SneakerHooknoseSWITCH <- read_csv("SneakerHooknoseSWITCH.csv") #Load data

SneakerHooknoseSWITCH<-SneakerHooknoseSWITCH[-c(24,38,55),] #Remove rows with
missing data

mod1<-lmer(Diff.VAP.SWITCHED ~ Diff.VAP.own + (1|MaleID)+(1|PAIR),data=Sneake
rHooknoseSWITCH)
summary(mod1)
```

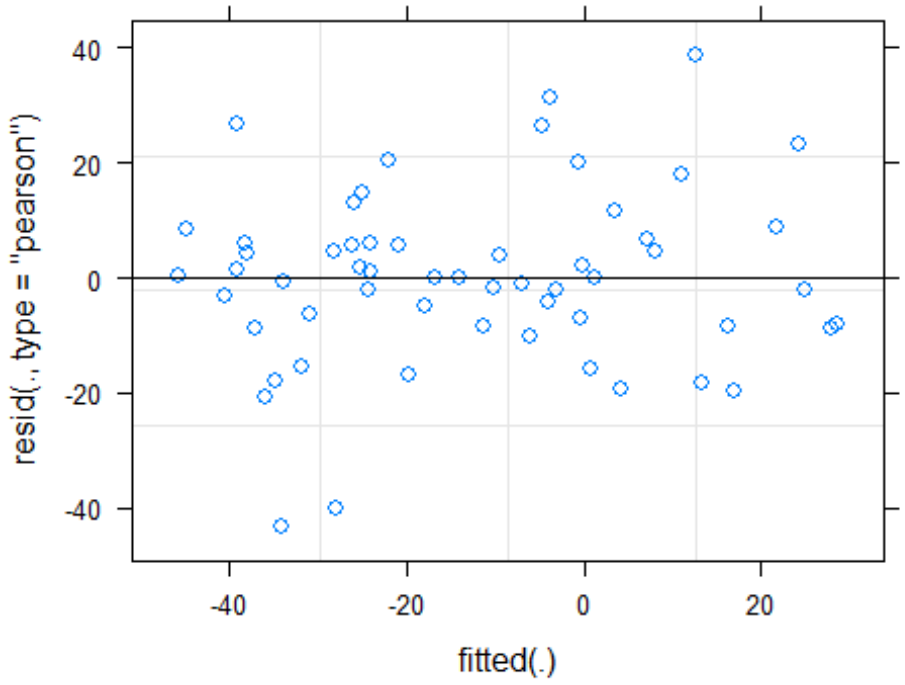
```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: Diff.VAP.SWITCHED ~ Diff.VAP.own + (1 | MaleID) + (1 | PAIR)
##    Data: SneakerHooknoseSWITCH
##
## REML criterion at convergence: 494.4
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -2.59931 -0.50032 -0.00341  0.36059  2.32730
##
## Random effects:
##  Groups   Name        Variance  Std.Dev.
##  PAIR     (Intercept) 1.244e-15 3.527e-08
##  MaleID   (Intercept) 1.956e+02 1.398e+01
##  Residual             2.786e+02 1.669e+01
## Number of obs: 57, groups:  PAIR, 30; MaleID, 11
##
## Fixed effects:
##             Estimate Std. Error       df t value Pr(>|t|)
## (Intercept)  -12.6170     4.7716   7.5400  -2.644  0.03108 *
```

```
## Diff.VAP.own   0.5456     0.1579  36.1600   3.454  0.00142 **
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr)
## Diff.VAP.wn 0.049
```

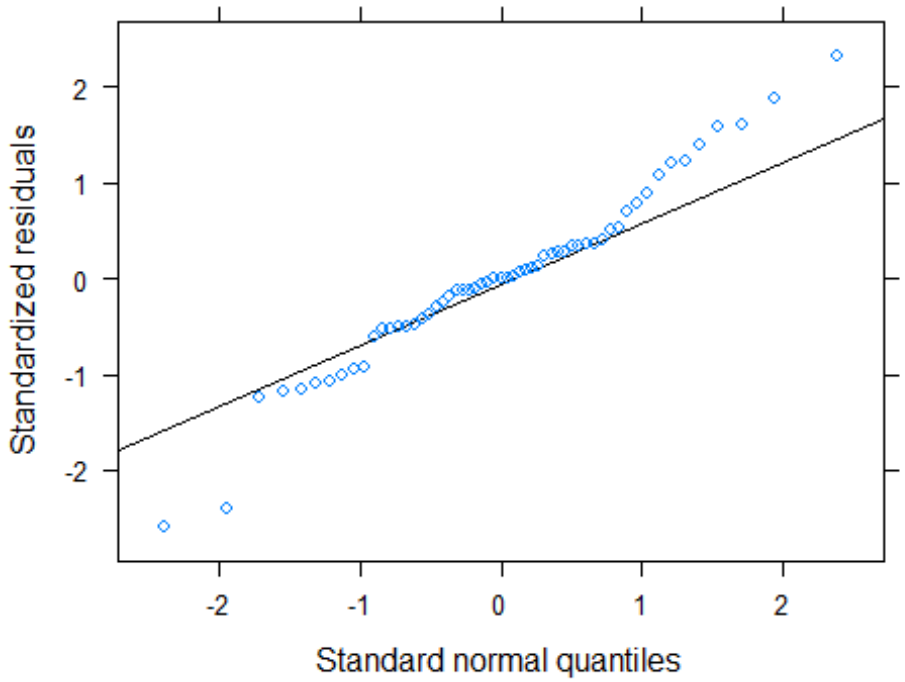
```
confint(mod1, level=0.95, method="Wald",oldNames=F) #generate 95%CI using Wal
d method
```

```
##                               2.5 %     97.5 %
## sd_(Intercept)|PAIR              NA         NA
## sd_(Intercept)|MaleID            NA         NA
## sigma                            NA         NA
## (Intercept)           -21.9692524 -3.2648390
## Diff.VAP.own            0.2360489  0.8551802
```

```
#Model diagnostics
plot1<-plot(mod1, results="hide", fig.show='hide') #plot residuals vs fitted
values to check for unequal variance
print(plot1)
```




```
qqmath(mod1) #check normality assumption
```




```
shapiro.test(resid(mod1))

##
##  Shapiro-Wilk normality test
##
## data:  resid(mod1)
## W = 0.97081, p-value = 0.183

rand(mod1)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID    8.7      1   0.003 **
## PAIR      0.0      1   1.000
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Reanalysis of data from Lewis and Pitcher 2017 Theriogenology

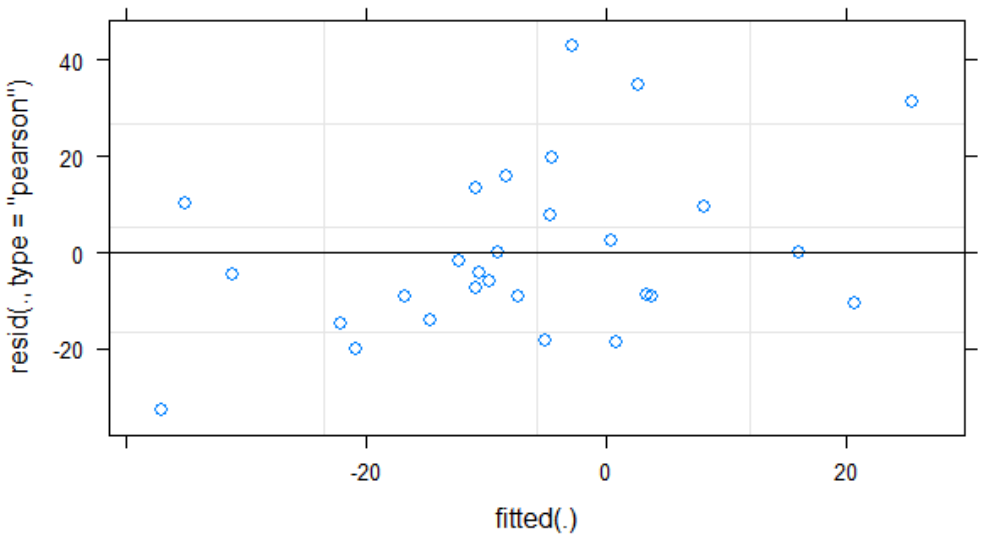
```
Lewis_and_Pitcher_data_VAP5s<-Lewis_and_Pitcher_data_VAP5s[-c(7,15,21,31),] #
Remove rows with missing data

modLPdata<-lmer(Diff.VAP.SWAP ~ Diff.VAP.own +(1|PAIR),data=Lewis_and_Pitcher
_data_VAP5s)

summary(modLPdata)
```

```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: Diff.VAP.SWAP ~ Diff.VAP.own + (1 | PAIR)
##    Data: Lewis_and_Pitcher_data_VAP5s
##
## REML criterion at convergence: 252.8
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.5959 -0.4696 -0.2136  0.4708  2.0962
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  PAIR     (Intercept) 186.2    13.65
##  Residual             417.4    20.43
## Number of obs: 28, groups:  PAIR, 15
##
## Fixed effects:
##              Estimate Std. Error      df t value Pr(>|t|)
## (Intercept)   -5.5183     5.2827 14.2970  -1.045  0.31355
## Diff.VAP.own   0.3425     0.1160 17.0750   2.954  0.00886 **
## ---
## Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
##
## Correlation of Fixed Effects:
##             (Intr)
## Diff.VAP.wn 0.105
```

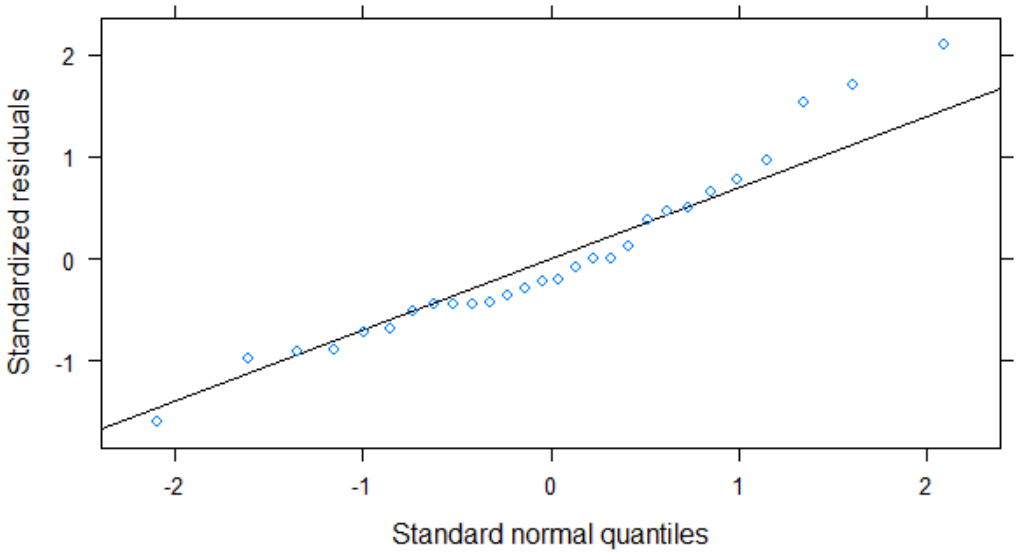
```
confint(modLPdata, level=0.95, method="Wald",oldNames=F) #generate 95%CI usin
g Wald method
```

```
##                             2.5 %    97.5 %
## sd_(Intercept)|PAIR            NA        NA
## sigma                          NA        NA
## (Intercept)         -15.8721974 4.8355571
## Diff.VAP.own          0.1152383 0.5698594
```

```
#Model diagnostics

plotmodLPdata<-plot(modLPdata, results="hide", fig.show='hide') #plot residua
ls vs fitted values to check for unequal variance

print(plotmodLPdata)
```




```
qqmath(modLPdata) #check normality assumption
```




```
shapiro.test(resid(modLPdata))
```

```
## Shapiro-Wilk normality test
##
## data:  resid(modLPdata)
## W = 0.94752, p-value = 0.1716
```

```
rand(modLPdata)#Test for significance of random predictor male ID
```

```
## Analysis of Random effects Table:
##      Chi.sq Chi.DF p.value
## PAIR   1.34      1     0.2
```

APPENDIX D:

PEAK INTEGRATION IN SKYLINE

MS1 full-scan features were associated with MS/MS identifications and were manually checked for appropriate integration using Skyline 3.7. Only peptides that could be reliably scored across samples were retained for further analyses. For 149 peptides, peaks could not be integrated in one sample and for 5 peptides peaks could not be integrated for two samples; these peptides were retained and for the samples in which they could not be scored were treated as missing data. Peptides were not retained if they had poor chromatography (Figure D.1) or were at low intensity/missing in 3 or more runs (Figure D.2). Where appropriate, shouldering peaks from a single transition were removed across all runs for that peptide (Figure D.3).

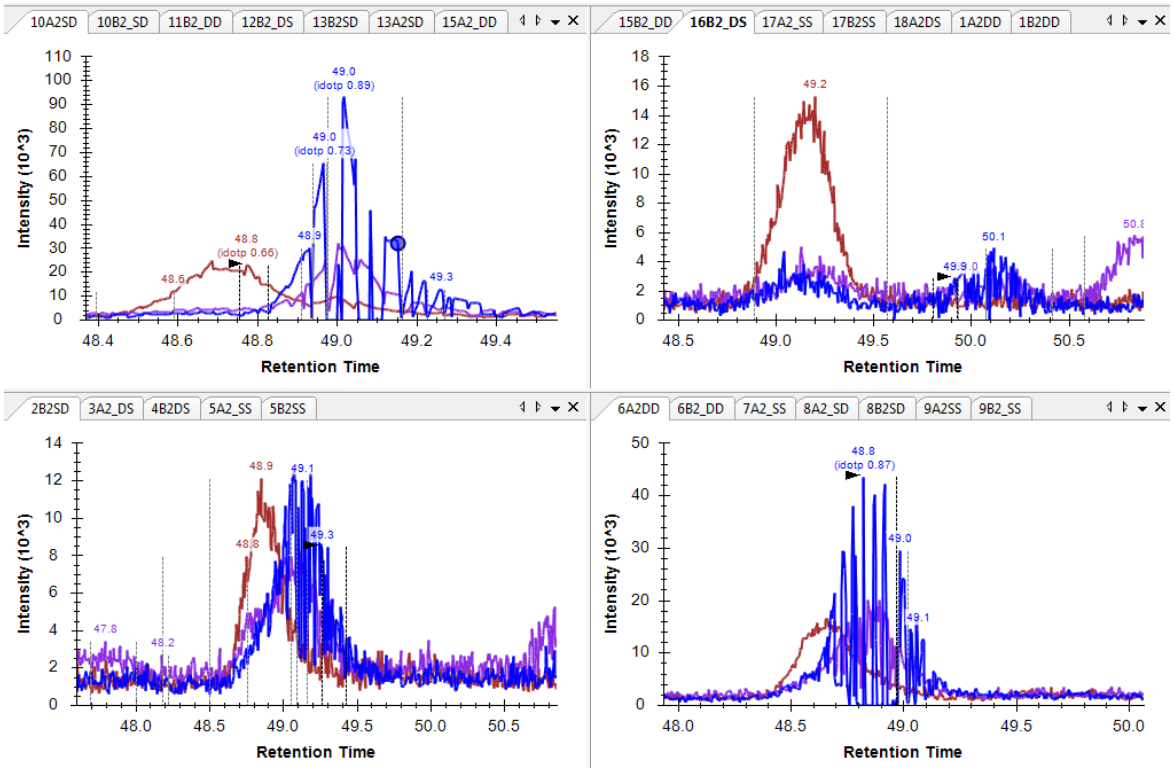


Figure D.1: Here is an example of poor chromatography, peptides with poor chromatography like this were removed from the data set because measurement of the area under these peaks is unreliable.

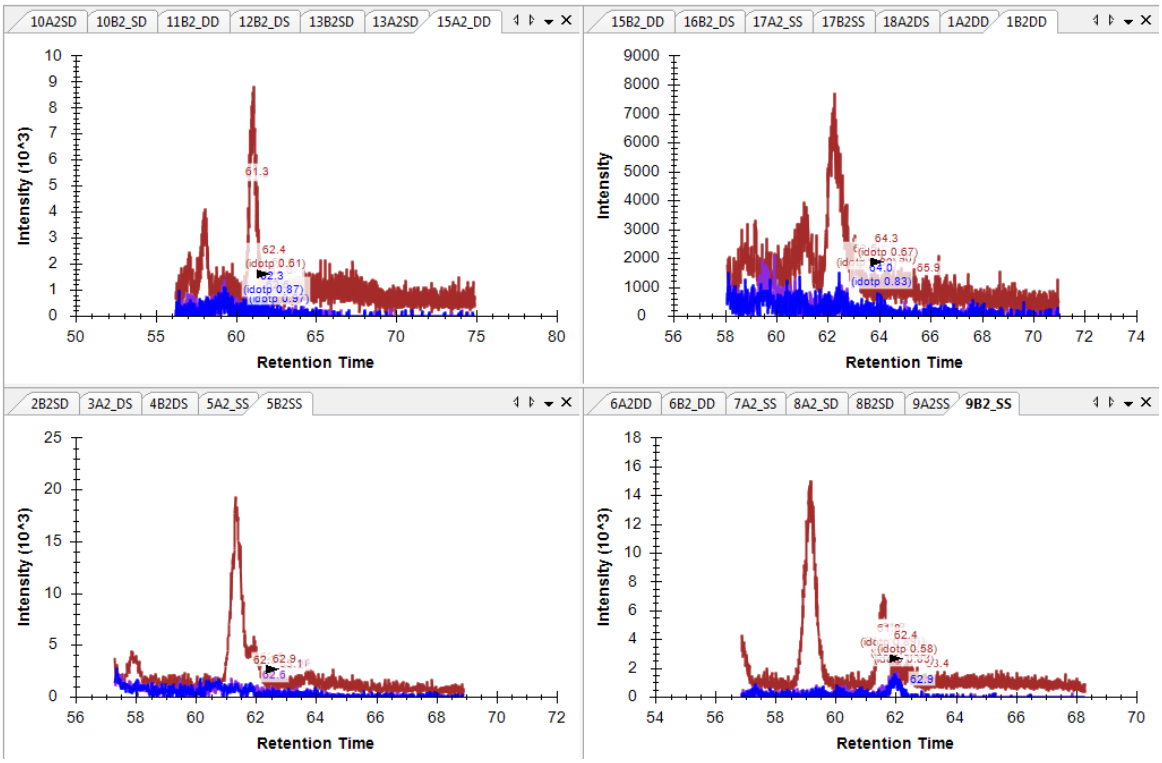


Figure D.2: Here the peak should be at approximately 62-64 minutes but it is at very low intensity/ not present so was not scored. In this case the red transition would also have had to be removed, see Figure D.3 below.

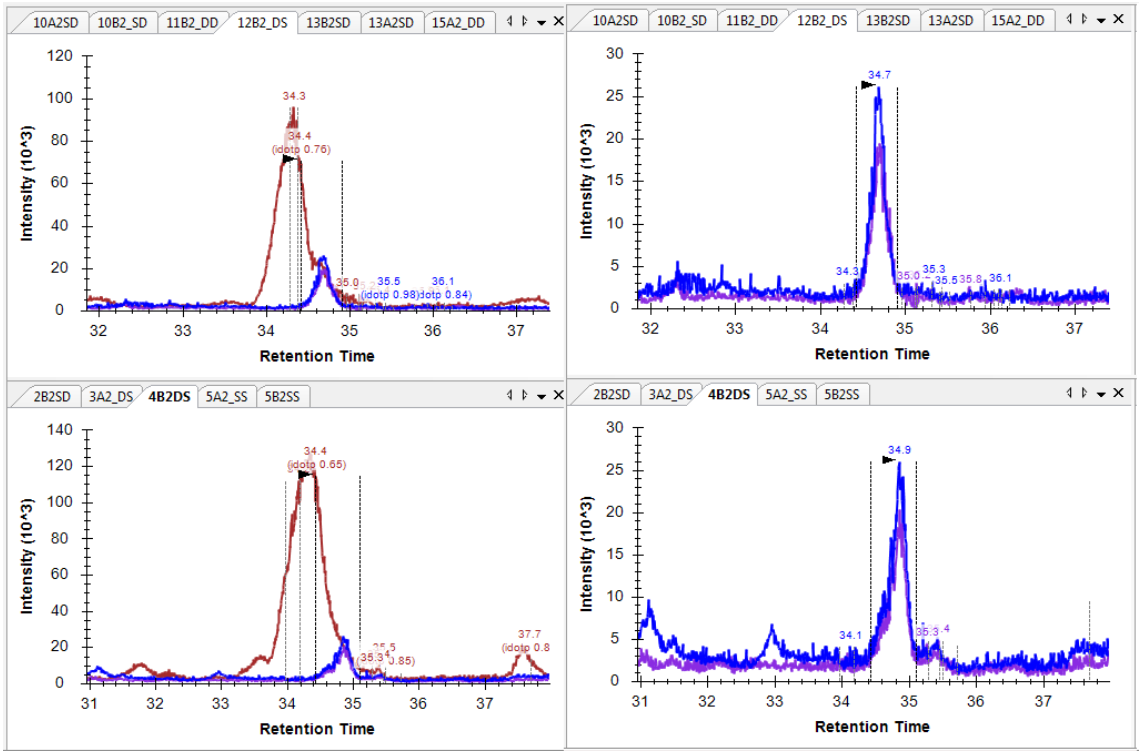


Figure D.3: The replicates shown on the left side are repeated on the right after the red transition was removed because the peak of interest was shouldered by a large peak from that transition. These measurements were retained in the data set, if I removed a transition then it was removed for all of the replicates for that peptide.

APPENDIX E:

CHAPTER FIVE: STATISTICAL ANALYSIS AND R CODE

CHAPTER 5 STATISTICAL ANALYSIS AND R CODE

CONTENTS PAGE

Packages used

```
library(lme4)
library(lmerTest)
library(lattice)
library(ggplot2)
library(MASS)
library(car)
library(LMERConvenienceFunctions)
library(multcomp)
library(lsmeans)
library(plyr)
library(scales)


proteins<-colnames(RelativeabundanceafterNormMSstats[,10:357]) #this calls a
character string for the 348 protein names
StageAonly<-RelativeabundanceafterNormMSstats[c(-5,-6,-7,-8,-9,-12,-15,-18,-1
9,-21,-22,-23,-24,-26,-32),]
StageBonly<-RelativeabundanceafterNormMSstats[c(-1,-2,-3,-4,-10,-11,-13,-14,-
16,-17,-20,-25,-27,-28,-29,-30,-31),]
```

MODELS COMPARING SPERM VELOCITY OR SPERM NUMBER BETWEEN DOMINANT AND SUBDOMINANT MALES

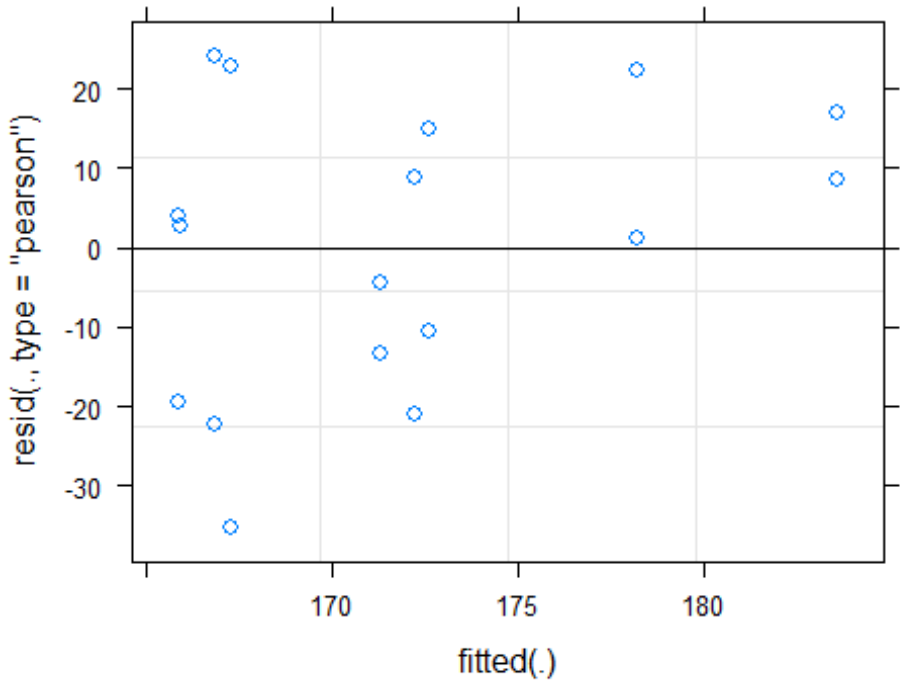
```
modVAPA<-lmer(VAP ~ Status + (1|Week),data=StageAonly)
summary(modVAPA)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: VAP ~ Status + (1 | Week)
##    Data: StageAonly
##
## REML criterion at convergence: 138
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.8054 -0.6792  0.1354  0.7687  1.2374
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  Week     (Intercept)  73.63    8.581
##  Residual             383.96   19.595
## Number of obs: 17, groups:  Week, 5
##
## Fixed effects:
##             Estimate Std. Error      df t value Pr(>|t|)
## (Intercept)  168.835      7.606   8.610  22.197  6.7e-09 ***
## StatusS        5.362      9.719  13.021   0.552     0.59
```

```
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##         (Intr)
## StatusS -0.588
```

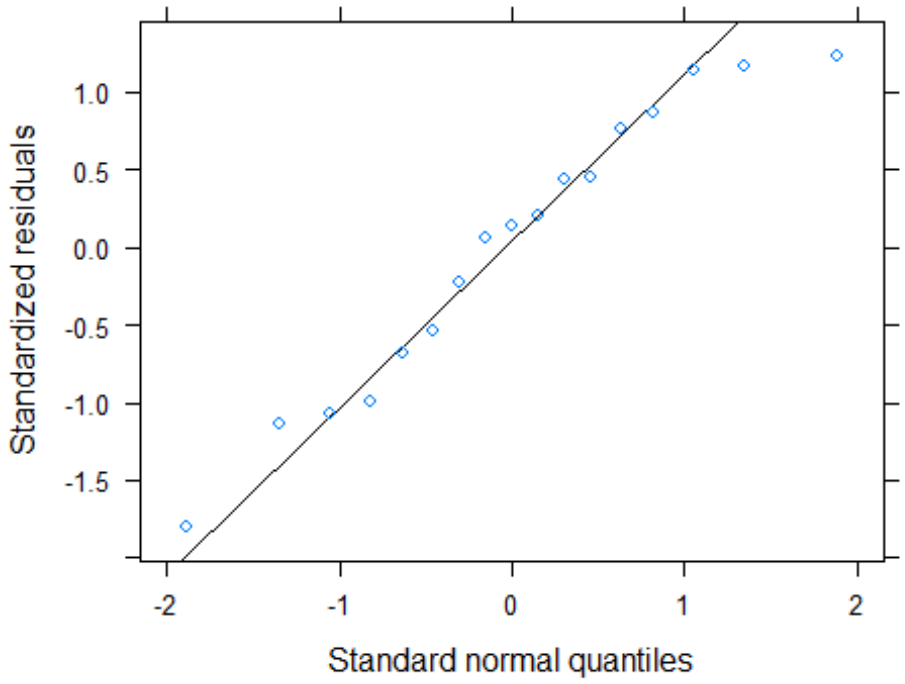
```
confint.merMod(modVAPA,level=0.95,method="Wald")
```

```
##                  2.5 %   97.5 %
## .sig01              NA       NA
## .sigma              NA       NA
## (Intercept) 153.92677 183.7424
## StatusS     -13.68679  24.4116
```

```
plot(modVAPA, results="hide", fig.show='hide')#Visual Check Variance assumpti
on
```




```
qqmath(modVAPA)#Visual Check Normality assumption
```



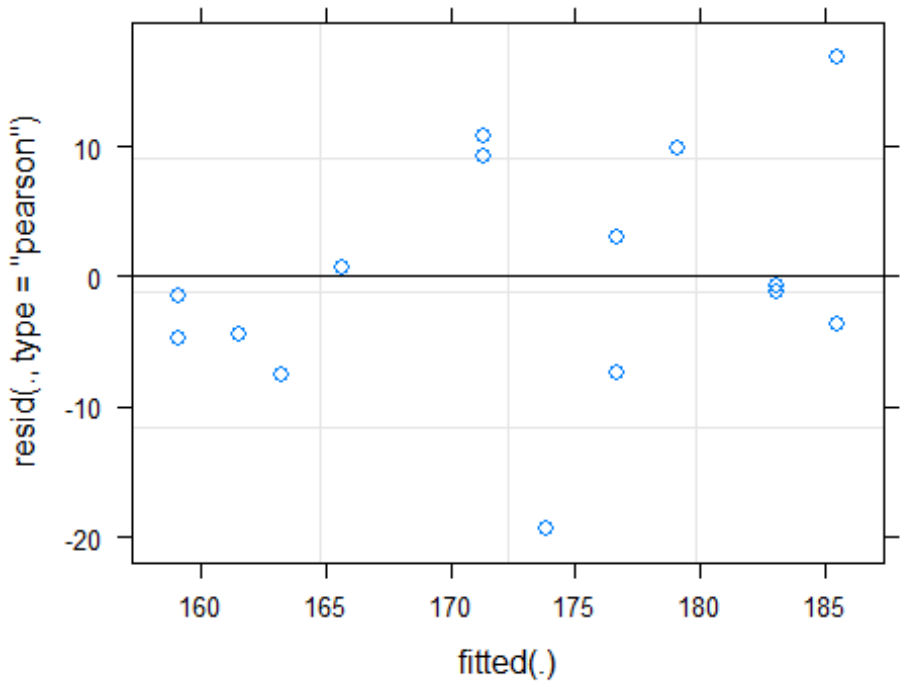

```
shapiro.test(resid(modVAPA))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
## data:  resid(modVAPA)
## W = 0.95092, p-value = 0.4712

rand(modVAPA)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##      Chi.sq Chi.DF p.value
## Week  0.467      1     0.5

modVAPB<-lmer(VAP ~ Status + (1|Week),data=StageBonly)
summary(modVAPB)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: VAP ~ Status + (1 | Week)
##    Data: StageBonly
##
## REML criterion at convergence: 108.2
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.8116 -0.4259 -0.1157  0.5740  1.5787
##
```

```
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  Week     (Intercept) 125.2    11.19
##  Residual             114.7    10.71
## Number of obs: 15, groups:  Week, 5
##
## Fixed effects:
##             Estimate Std. Error      df t value Pr(>|t|)
## (Intercept)  170.664      6.177   5.391  27.630 5.14e-07 ***
## StatusS        2.457      5.705   9.254   0.431    0.677
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##         (Intr)
## StatusS -0.369
```

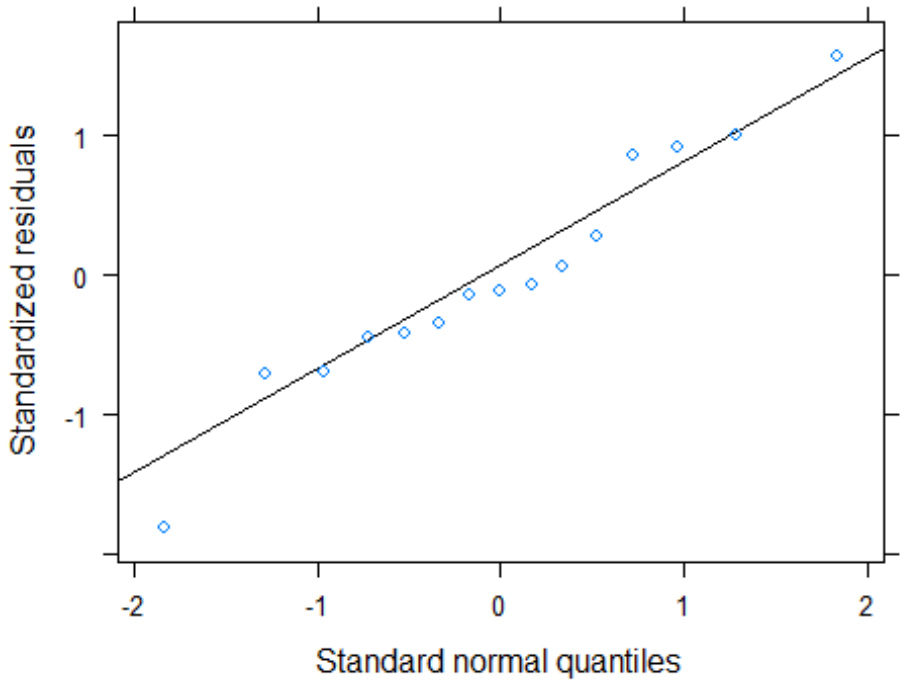
```
confint.merMod(modVAPB,level=0.95,method="Wald")
```

```
##                   2.5 %    97.5 %
## .sig01               NA        NA
## .sigma               NA        NA
## (Intercept) 158.557993 182.77047
## StatusS      -8.724226  13.63748
```

```
plot(modVAPB, results="hide", fig.show='hide')#Visual Check Variance assumpti
on
```

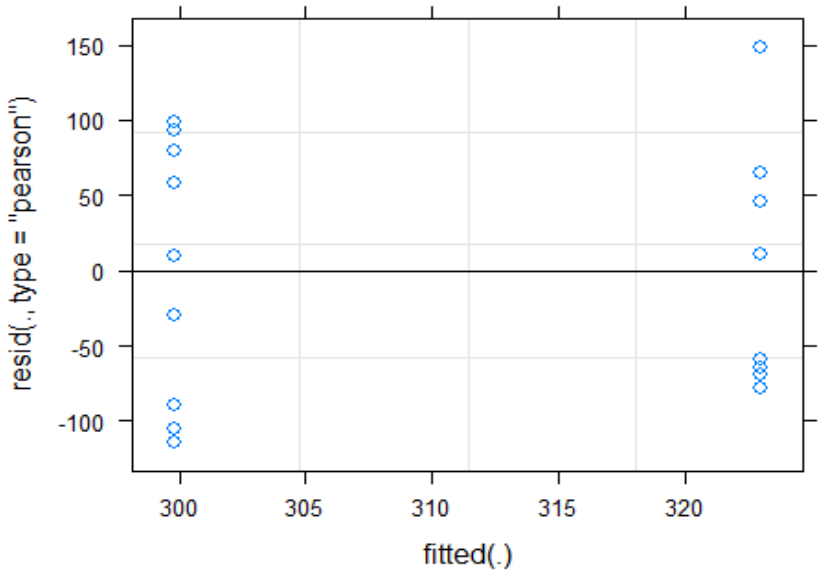


```
qqmath(modVAPB)#Visual Check Normality assumption
```

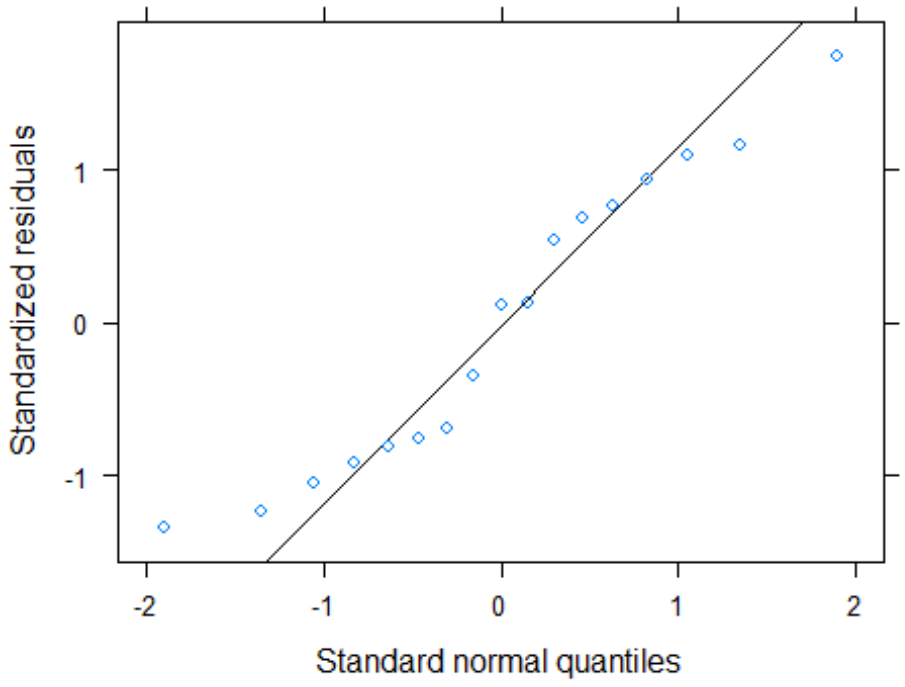



```
shapiro.test(resid(modVAPB))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
## data:  resid(modVAPB)
## W = 0.96052, p-value = 0.7015

rand(modVAPB)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##      Chi.sq Chi.DF p.value
## Week   3.56      1    0.06 .
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

modcountA<-lmer(SpermCount ~ Status + (1|Week), data=StageAonly)
summary(modcountA)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: SpermCount ~ Status + (1 | Week)
##    Data: StageAonly
##
## REML criterion at convergence: 180.2
##
```

```
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.3475 -0.8101  0.1200  0.7631  1.7493
##
## Random effects:
##  Groups   Name        Variance  Std.Dev.
##  Week     (Intercept) 3.038e-12 1.743e-06
##  Residual             7.255e+03 8.518e+01
## Number of obs: 17, groups:  Week, 5
##
## Fixed effects:
##             Estimate Std. Error     df t value Pr(>|t|)
## (Intercept)   299.78      28.39  14.99  10.558 2.45e-08 ***
## StatusS        23.22      41.39  14.99   0.561    0.583
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##         (Intr)
## StatusS -0.686

confint.merMod(modcountA,level=0.95,method="Wald")

##                 2.5 %   97.5 %
## .sig01             NA       NA
## .sigma             NA       NA
## (Intercept) 244.12913 355.4264
## StatusS     -57.89893 104.3434

plot(modcountA, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```



```
qqmath(modcountA)#Visual Check Normality assumption
```



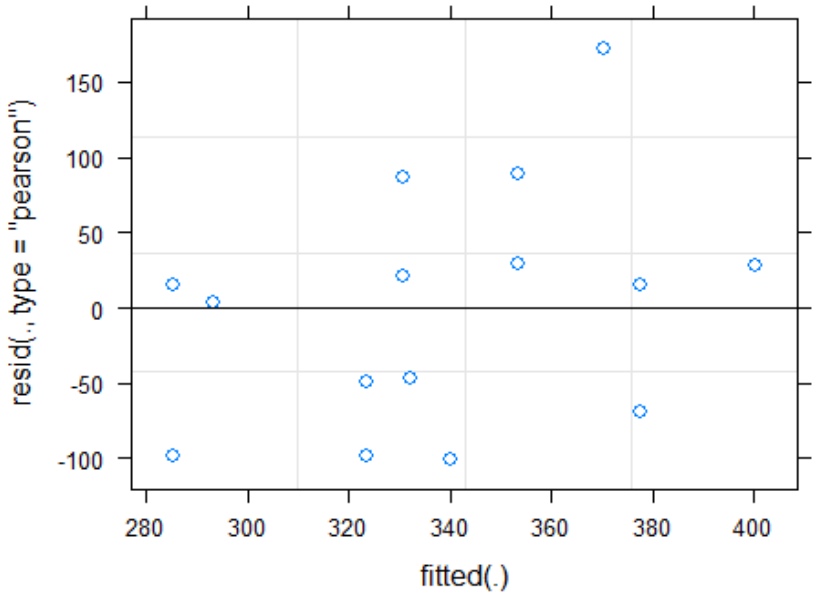

```
shapiro.test(resid(modcountA))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
## data:  resid(modcountA)
## W = 0.93526, p-value = 0.2662

rand(modcountA)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## Week 2.84e-14      1       1

modcountB<-lmer(SpermCount ~ Status + (1|Week), data=StageBonly)
summary(modcountB)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: SpermCount ~ Status + (1 | Week)
##    Data: StageBonly
##
## REML criterion at convergence: 159.3
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
```

```
## -1.1559 -0.6733  0.1703  0.3322  1.9736
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  Week     (Intercept) 1883     43.39
##  Residual             7659     87.52
## Number of obs: 15, groups:  Week, 5
##
## Fixed effects:
##             Estimate Std. Error      df t value Pr(>|t|)
## (Intercept)  317.119     35.211   6.634   9.006 5.86e-05 ***
## StatusS       46.971     46.407   9.106   1.012    0.338
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##         (Intr)
## StatusS -0.526
```

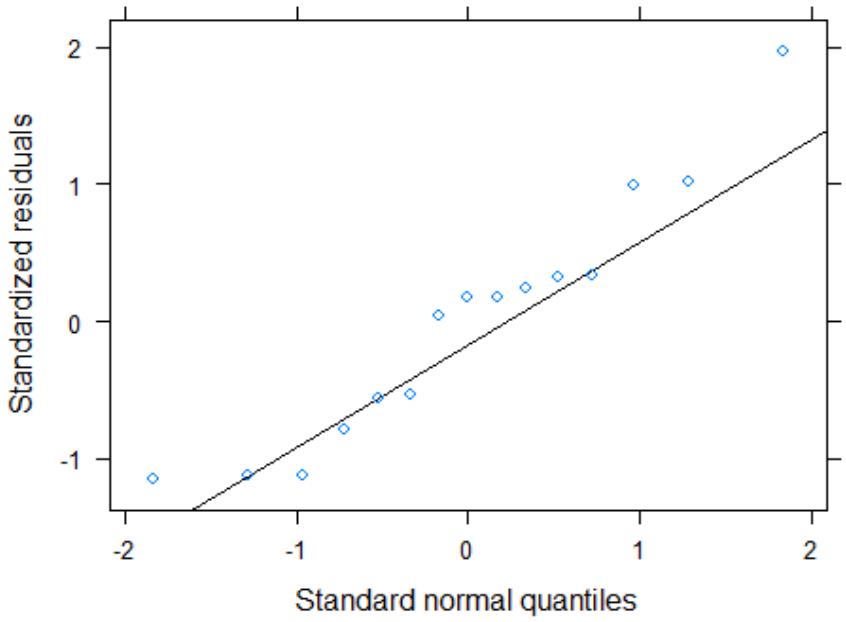
```
confint.merMod(modcountB,level=0.95,method="Wald")
```

```
##                 2.5 %   97.5 %
## .sig01             NA       NA
## .sigma             NA       NA
## (Intercept) 248.10571 386.1315
## StatusS     -43.98418 137.9272
```

```
plot(modcountB, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```



```
qqmath(modcountB)#Visual Check Normality assumption
```




```
shapiro.test(resid(modcountB))#Test Check Normality assumption
```

```
##
##  Shapiro-Wilk normality test
##
## data:  resid(modcountB)
## W = 0.9335, p-value = 0.3076
```

```
rand(modcountB)#Test for significance of random predictor male ID
```

```
## Analysis of Random effects Table:
##      Chi.sq Chi.DF p.value
## Week  0.395      1     0.5
```

COMPARING DOMINANT AND SUBDOMINANT MALES STAGE 1: models with status as significant predictor are shown below. VAP and sperm number were included as fixed effects in these models

```
proteins[[7]]
```

```
## [1] "H1_ONCMY"
```

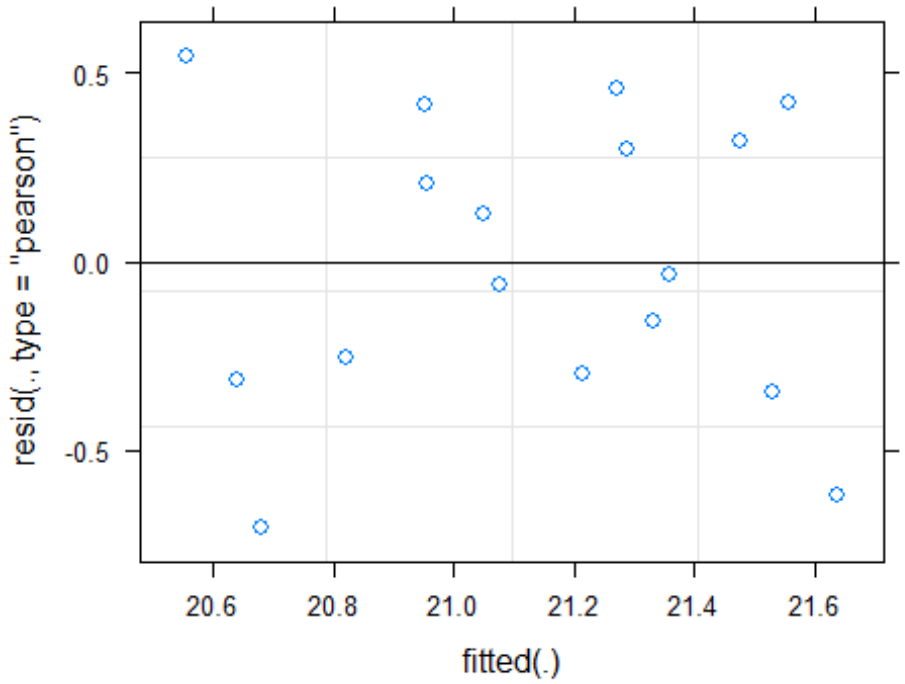
```
STAGEAMODELP7<-lmer(H1_ONCMY ~ rescale(VAP) + rescale(SpermCount) + Status +
                      + (1|Week),data=StageAonly)
summary(STAGEAMODELP7)
```

```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: H1_ONCMY ~ VAP + SpermCount + Status + +(1 | Week)
##    Data: StageAonly
##
## REML criterion at convergence: 39.6
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.64765 -0.69514 -0.08414  0.74070  1.27466
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  Week     (Intercept) 0.0000   0.0000
##  Residual             0.1845   0.4296
## Number of obs: 17, groups:  Week, 5
##
## Fixed effects:
##              Estimate Std. Error        df t value Pr(>|t|)
## (Intercept) 22.272320   1.055194 13.000000  21.107 1.92e-11 ***
## VAP         -0.003339   0.005312 13.000000  -0.629   0.5406
## SpermCount  -0.002669   0.001324 13.000000  -2.016   0.0649 .
## StatusS      0.577854   0.214001 13.000000   2.700   0.0182 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn
## VAP         -0.919
## SpermCount -0.529  0.180
## StatusS     0.117 -0.169 -0.169
```

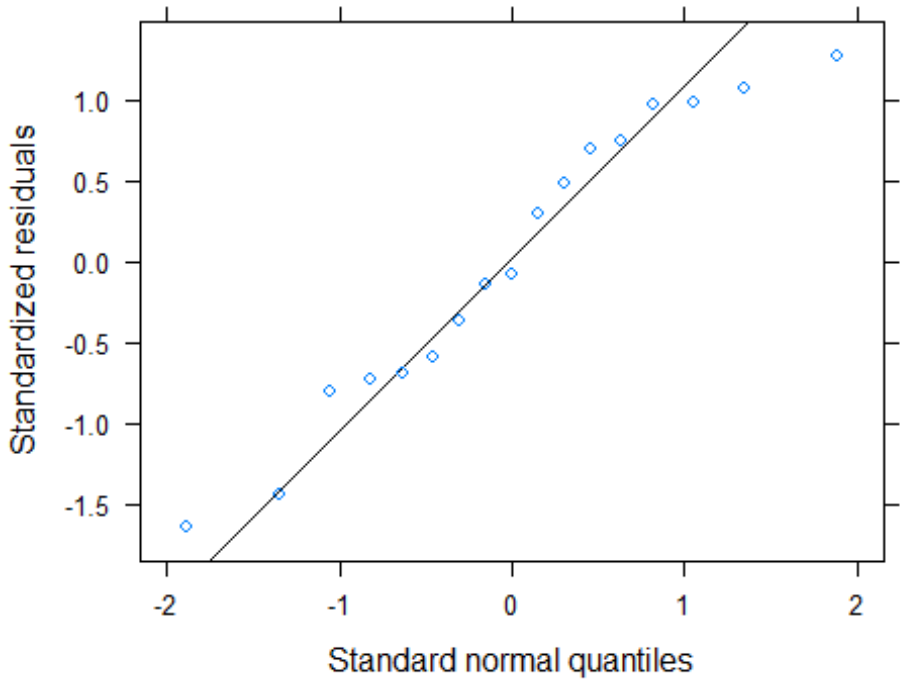
```
confint.merMod(STAGEAMODELP7,level=0.95,method="Wald")
```

```
##                    2.5 %        97.5 %
## .sig01                NA            NA
## .sigma                NA            NA
## (Intercept) 20.204177286  2.434046e+01
## VAP         -0.013750100  7.072826e-03
```

```
## SpermCount  -0.005263075 -7.442578e-05
## StatusS      0.158419133  9.972883e-01

plot(STAGEAMODELP7, results="hide", fig.show='hide')#Visual Check Variance as
sumption
```

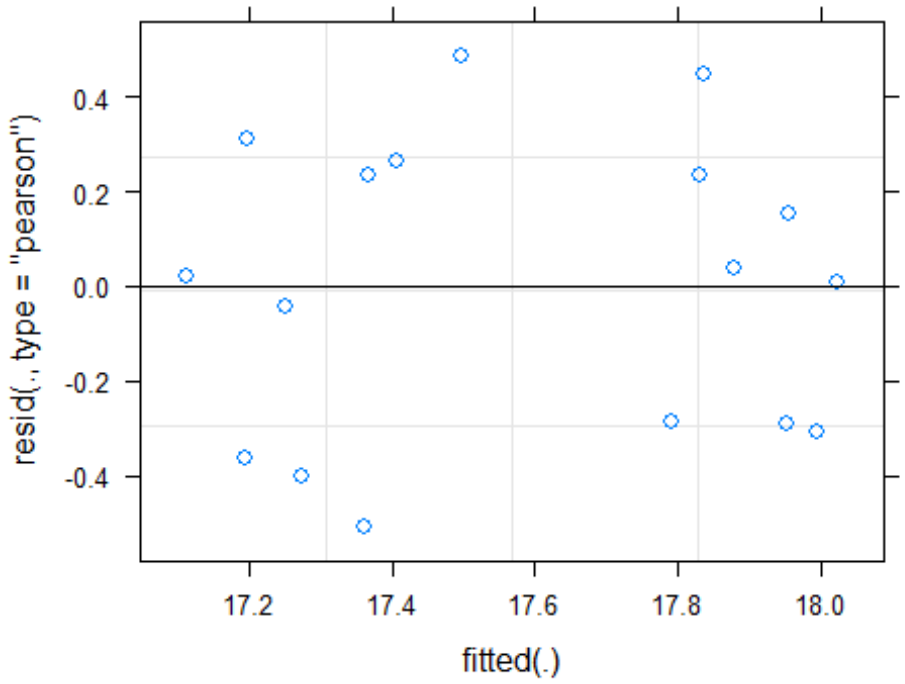



```
qqmath(STAGEAMODELP7)#Visual Check Normality assumption
```

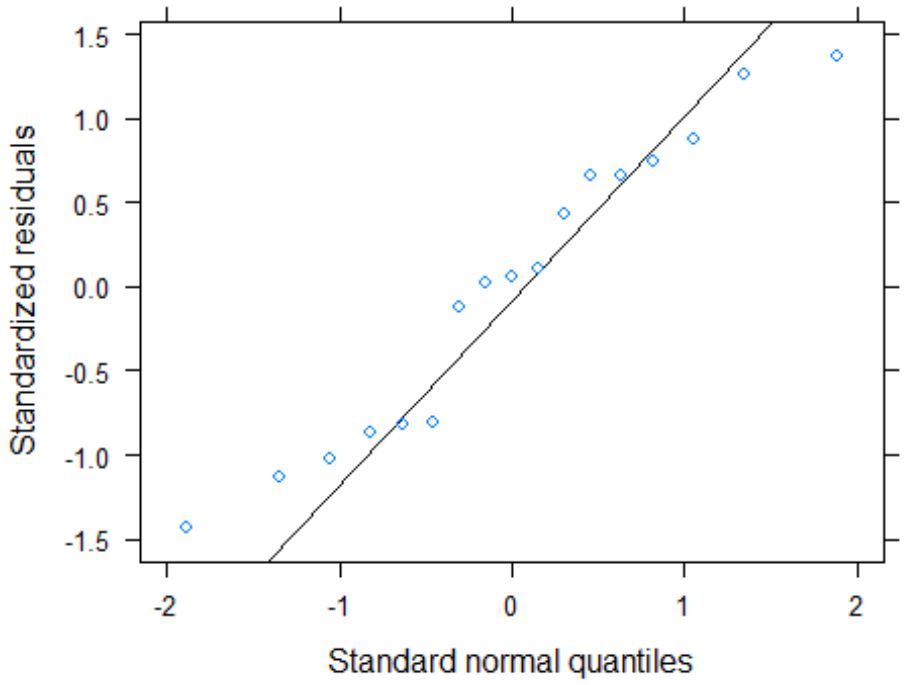



```
shapiro.test(resid(STAGEAMODELP7))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
## data:  resid(STAGEAMODELP7)
## W = 0.94531, p-value = 0.3866

rand(STAGEAMODELP7)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## Week 4.97e-14      1       1

proteins[[41]]

## [1] "B5DGT2_SALSA"

STAGEAMODELP41<-lmer(B5DGT2_SALSA ~ rescale(VAP) + rescale(SpermCount) + Stat
us +
                       + (1|Week),data=StageAonly)
summary(STAGEAMODELP41)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: B5DGT2_SALSA ~ VAP + SpermCount + Status + +(1 | Week)
##    Data: StageAonly
##
```

```
## REML criterion at convergence: 35.2
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.43881 -0.82268  0.06051  0.65823  1.37801
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  Week     (Intercept) 0.00774  0.08798
##  Residual             0.12541  0.35414
## Number of obs: 17, groups:  Week, 5
##
## Fixed effects:
##              Estimate Std. Error        df t value Pr(>|t|)
## (Intercept) 18.216242   0.895036 12.983000  20.353 3.12e-11 ***
## VAP         -0.004460   0.004530 12.761000  -0.984  0.34318
## SpermCount  -0.000551   0.001107 12.258000  -0.498  0.62732
## StatusS      0.653189   0.178333 11.941000   3.663  0.00328 **
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##            (Intr) VAP    SprmCn
## VAP        -0.921
## SpermCount -0.521  0.177
## StatusS     0.126 -0.171 -0.184

confint.merMod(STAGEAMODELP41,level=0.95,method="Wald")

##                    2.5 %       97.5 %
## .sig01                NA           NA
## .sigma                NA           NA
## (Intercept) 16.462003670 19.970480408
## VAP         -0.013338952  0.004419207
## SpermCount  -0.002719592  0.001617624
## StatusS      0.303662394  1.002714817

plot(STAGEAMODELP41, results="hide", fig.show='hide')#Visual Check Variance a
ssumption
```




```
qqmath(STAGEAMODELP41)#Visual Check Normality assumption
```




```
shapiro.test(resid(STAGEAMODELP41))#Test Check Normality assumption
```

```
##
##  Shapiro-Wilk normality test
##
## data:  resid(STAGEAMODELP41)
## W = 0.9439, p-value = 0.3673
```

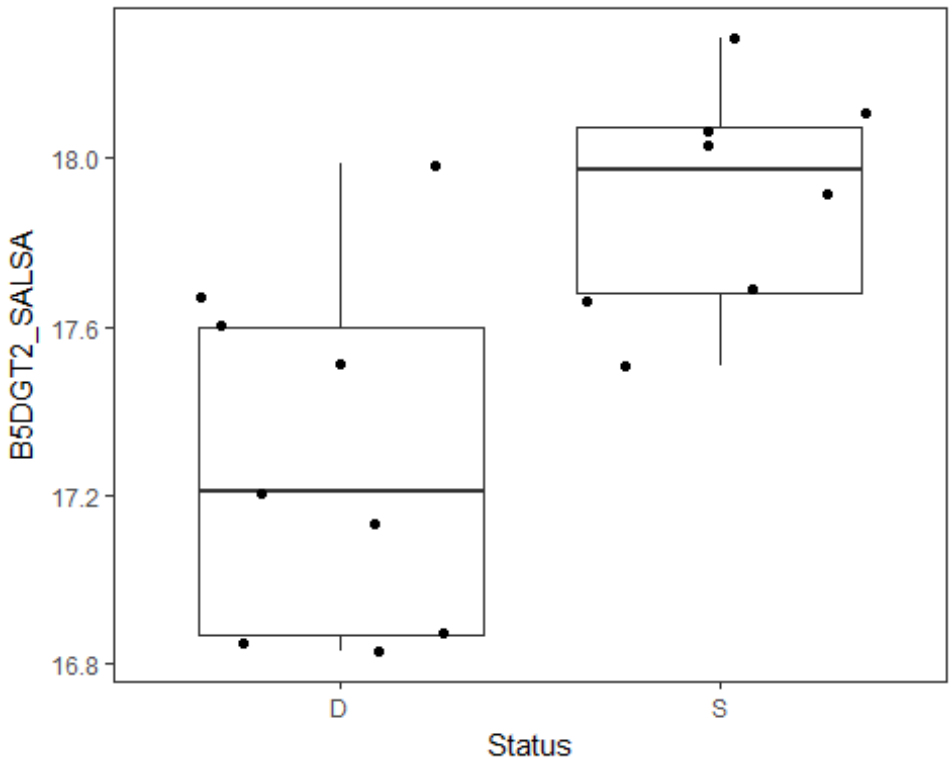
```
rand(STAGEAMODELP41)#Test for significance of random predictor male ID
```

```
## Analysis of Random effects Table:
##      Chi.sq Chi.DF p.value
## Week 0.0622      1     0.8
```

```
summarySE(data=StageAonly, measurevar = "B5DGT2_SALSA", groupvars = "Status",
conf.interval = .095)
```

```
##   Status N B5DGT2_SALSA        sd         se         ci
## 1      D 9     17.29610 0.4154710 0.13849034 0.01705978
## 2      S 8     17.90786 0.2649852 0.09368642 0.01159267
```

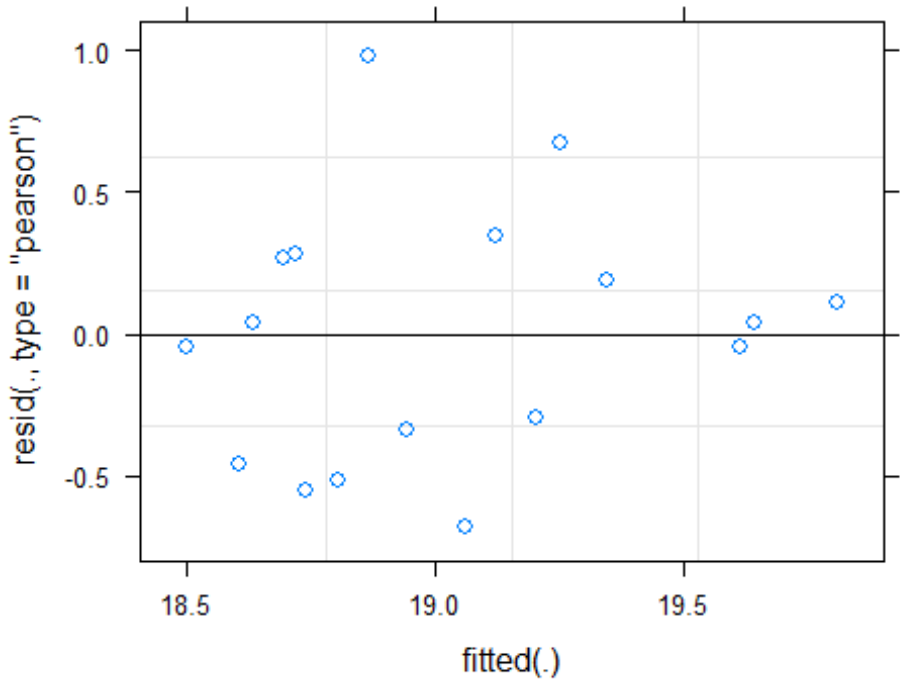
```
#Plot by status
ggplot(data=StageAonly, aes(x=Status, y=B5DGT2_SALSA)) +
  geom_boxplot()+ geom_jitter()+ theme_bw()+
  theme(panel.grid.minor=element_blank(),panel.grid.major=element_blank())
```



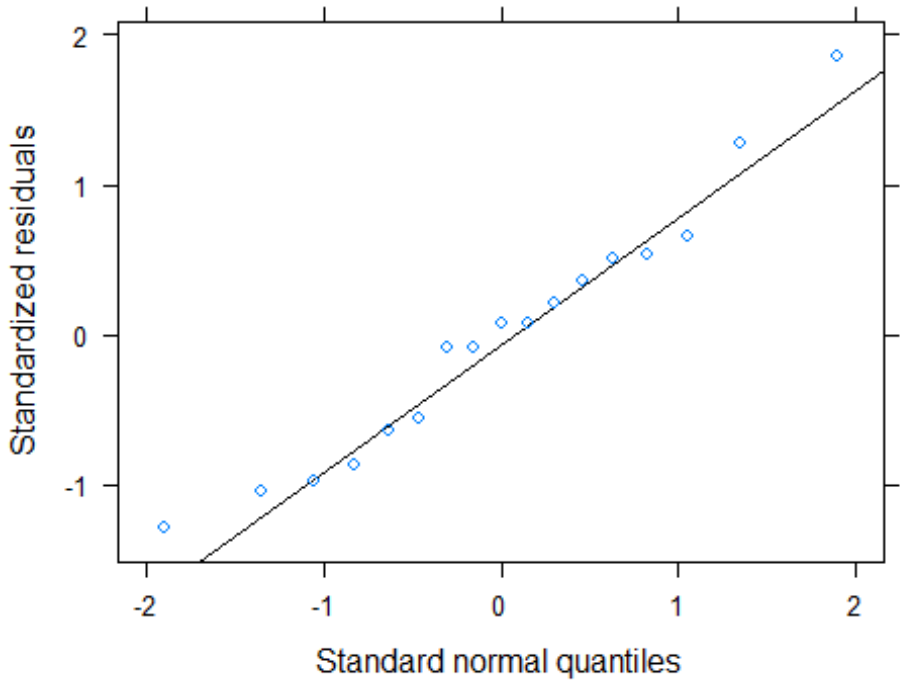

```
proteins[[44]]
```

```
## [1] "B5DGU8_SALSA"
```

```
STAGEAMODELP44<-lmer(B5DGU8_SALSA ~ rescale(VAP) + rescale(SpermCount) + Stat
us +
                       + (1|Week),data=StageAonly)
summary(STAGEAMODELP44)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: B5DGU8_SALSA ~ VAP + SpermCount + Status + +(1 | Week)
##    Data: StageAonly
##
## REML criterion at convergence: 46.9
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.2933 -0.6407  0.0743  0.5086  1.8632
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  Week     (Intercept) 0.06338  0.2517
##  Residual             0.27784  0.5271
## Number of obs: 17, groups:  Week, 5
##
## Fixed effects:
##              Estimate Std. Error        df t value Pr(>|t|)
## (Intercept) 17.029217   1.395317 12.645000  12.205  2.3e-08 ***
## VAP          0.011065   0.007115 12.900000   1.555   0.1441
## SpermCount   0.001428   0.001685 10.782000   0.848   0.4151
## StatusS     -0.689372   0.270731 10.580000  -2.546   0.0279 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##            (Intr) VAP    SprmCn
## VAP        -0.923
## SpermCount -0.509  0.173
## StatusS     0.139 -0.171 -0.209

confint.merMod(STAGEAMODELP44,level=0.95,method="Wald")

##                    2.5 %       97.5 %
## .sig01                NA           NA
## .sigma                NA           NA
## (Intercept) 14.294445732 19.763988739
## VAP         -0.002880843  0.025011366
## SpermCount  -0.001874326  0.004730937
## StatusS     -1.219994855 -0.158748747

plot(STAGEAMODELP44, results="hide", fig.show='hide')#Visual Check Variance a
ssumption
```




```
qqmath(STAGEAMODELP44)#Visual Check Normality assumption
```



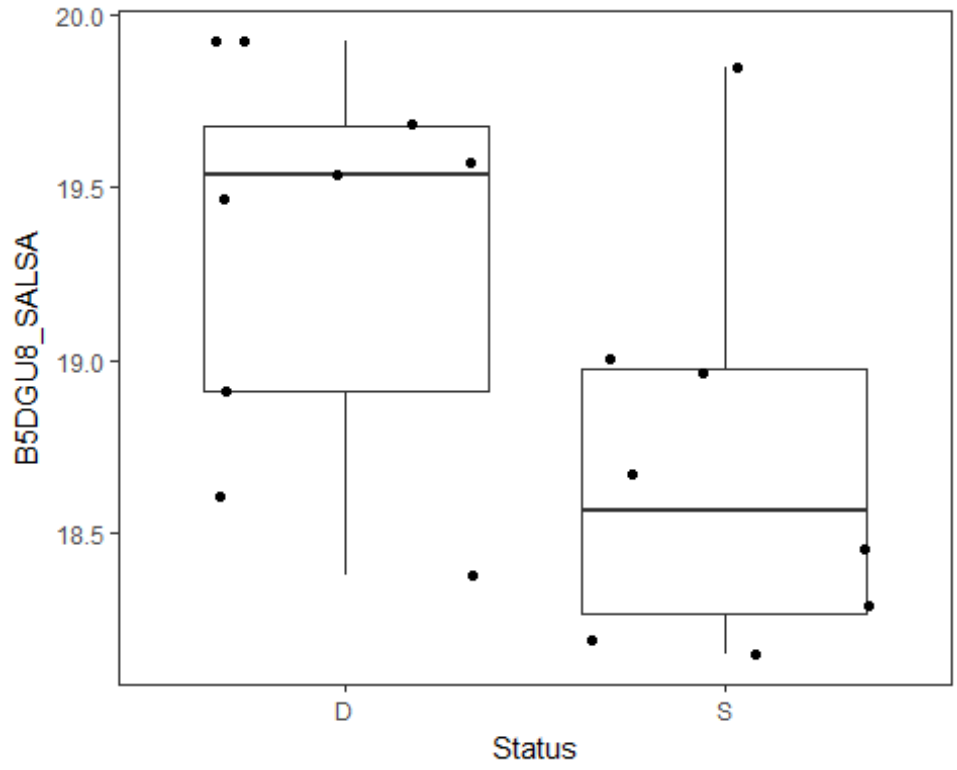

```
shapiro.test(resid(STAGEAMODELP44))#Test Check Normality assumption
```

```
##
##  Shapiro-Wilk normality test
##
## data:  resid(STAGEAMODELP44)
## W = 0.9645, p-value = 0.7169
```

```
rand(STAGEAMODELP44)#Test for significance of random predictor male ID
```

```
## Analysis of Random effects Table:
##      Chi.sq Chi.DF p.value
## Week  0.291      1     0.6
```

```
#Plot by status
ggplot(data=StageAonly, aes(x=Status, y=B5DGU8_SALSA)) +
  geom_boxplot()+ geom_jitter()+ theme_bw()+
  theme(panel.grid.minor=element_blank(),panel.grid.major=element_blank())
```




```
proteins[[73]]
```

```
## [1] "B5X1X1_SALSA"
```

```
STAGEAMODELP73<-lmer(B5X1X1_SALSA ~ rescale(VAP) + rescale(SpermCount) + Stat
us + (1|Week),data=StageAonly)
summary(STAGEAMODELP73)
```

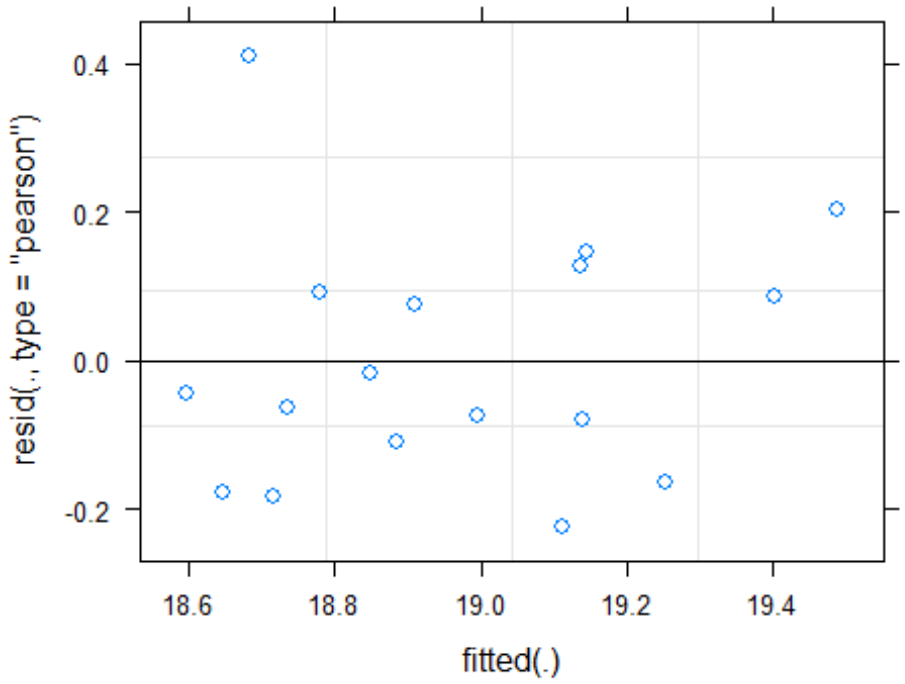
```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: B5X1X1_SALSA ~ VAP + SpermCount + Status + +(1 | Week)
```

```
##    Data: StageAonly
##
## REML criterion at convergence: 24.1
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.1011 -0.5404 -0.2170  0.4501  2.0103
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  Week     (Intercept) 0.02423  0.1557
##  Residual             0.04182  0.2045
## Number of obs: 17, groups:  Week, 5
##
## Fixed effects:
##               Estimate Std. Error         df t value Pr(>|t|)
## (Intercept) 20.5422267  0.5661413 11.4150000  36.285 3.76e-13 ***
## VAP         -0.0058584  0.0028999 11.5370000  -2.020   0.0672 .
## SpermCount  -0.0013451  0.0006686  9.7710000  -2.012   0.0726 .
## StatusS     -0.3058856  0.1073284  9.7860000  -2.850   0.0176 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn
## VAP         -0.922
## SpermCount -0.495  0.168
## StatusS     0.149 -0.168 -0.236
```

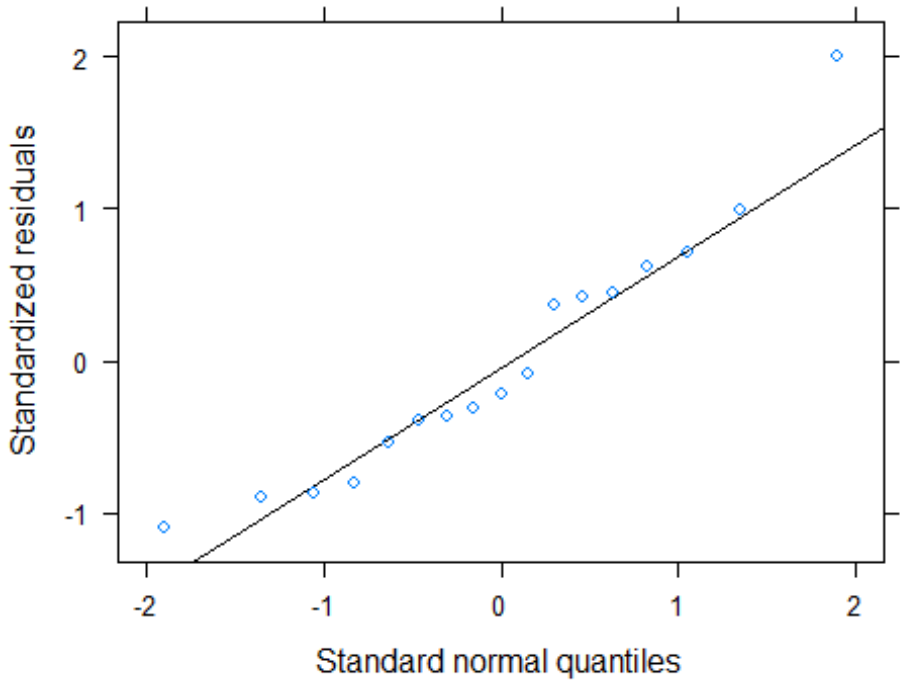
```
confint.merMod(STAGEAMODELP73,level=0.95,method="Wald")
```

```
##                    2.5 %        97.5 %
## .sig01                NA            NA
## .sigma                NA            NA
## (Intercept) 19.43261017  2.165184e+01
## VAP         -0.01154199 -1.747594e-04
## SpermCount  -0.00265549 -3.468444e-05
## StatusS     -0.51624533 -9.552588e-02
```

```
plot(STAGEAMODELP73, results="hide", fig.show='hide')#Visual Check Variance a
ssumption
```




```
qqmath(STAGEAMODELP73)#Visual Check Normality assumption
```



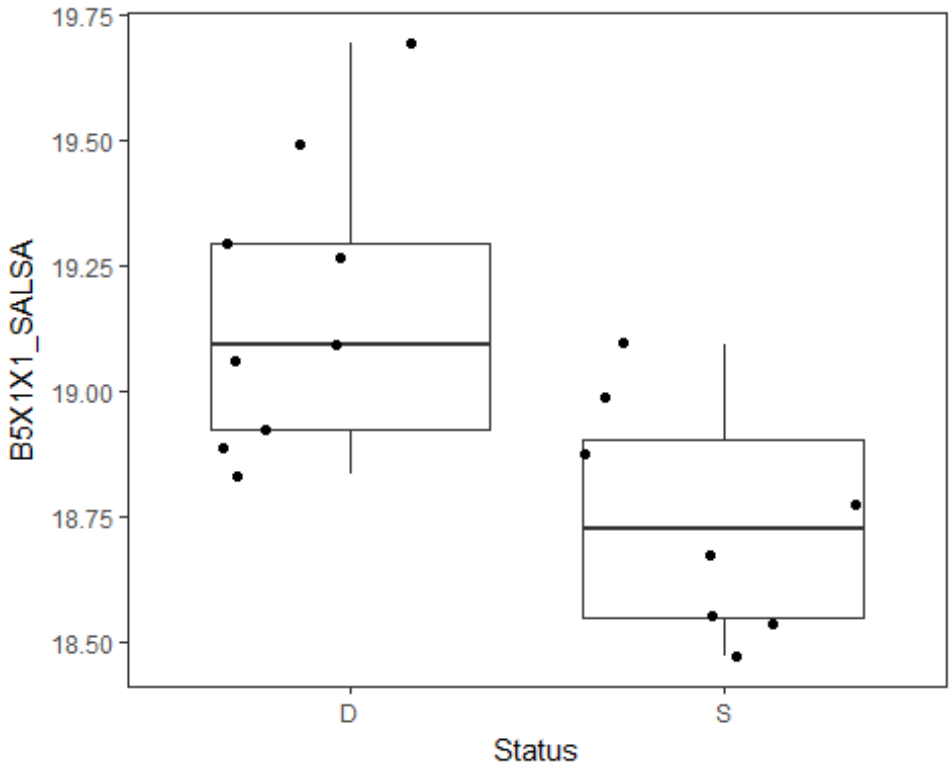

```
shapiro.test(resid(STAGEAMODELP73))#Test Check Normality assumption
```

```
##
##  Shapiro-Wilk normality test
##
## data:  resid(STAGEAMODELP73)
## W = 0.93851, p-value = 0.301
```

```
rand(STAGEAMODELP73)#Test for significance of random predictor male ID
```

```
## Analysis of Random effects Table:
##      Chi.sq Chi.DF p.value
## Week  0.899      1     0.3
```

```
#Plot by status
ggplot(data=StageAonly, aes(x=Status, y=B5X1X1_SALSA)) +
  geom_boxplot()+ geom_jitter()+ theme_bw()+
  theme(panel.grid.minor=element_blank(),panel.grid.major=element_blank())
```




```
proteins[[79]]
```

```
## [1] "B5X2I6_SALSA"
```

```
STAGEAMODELP79<-lmer(B5X2I6_SALSA ~ rescale(VAP) + rescale(SpermCount) Status
+
                       + (1|Week),data=StageAonly)
summary(STAGEAMODELP79)
```

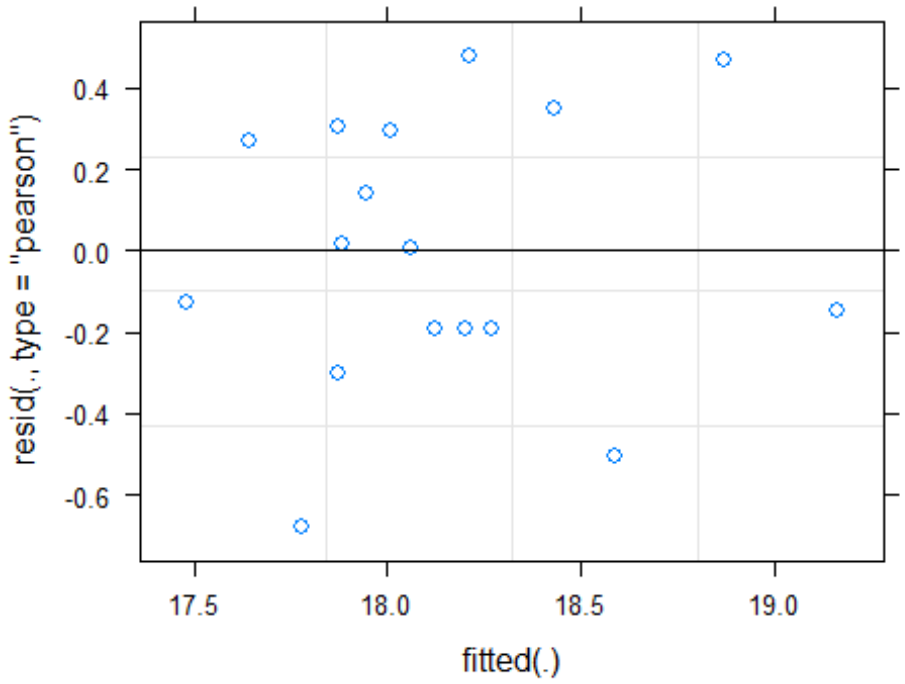
```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
```

```
## Formula: B5X2I6_SALSA ~ VAP + SpermCount + Status + +(1 | Week)
##    Data: StageAonly
##
## REML criterion at convergence: 38.7
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.73850 -0.48594  0.01629  0.75089  1.22873
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  Week     (Intercept) 0.02578  0.1606
##  Residual             0.15331  0.3915
## Number of obs: 17, groups:  Week, 5
##
## Fixed effects:
##              Estimate Std. Error        df t value Pr(>|t|)
## (Intercept) 14.887907   1.022785 12.868000  14.556 2.28e-09 ***
## VAP          0.013695   0.005206 12.999000   2.631   0.0208 *
## SpermCount   0.003594   0.001243 11.162000   2.891   0.0145 *
## StatusS     -0.489505   0.199909 10.885000  -2.449   0.0325 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##            (Intr) VAP    SprmCn
## VAP        -0.923
## SpermCount -0.512  0.174
## StatusS     0.136 -0.172 -0.201
```

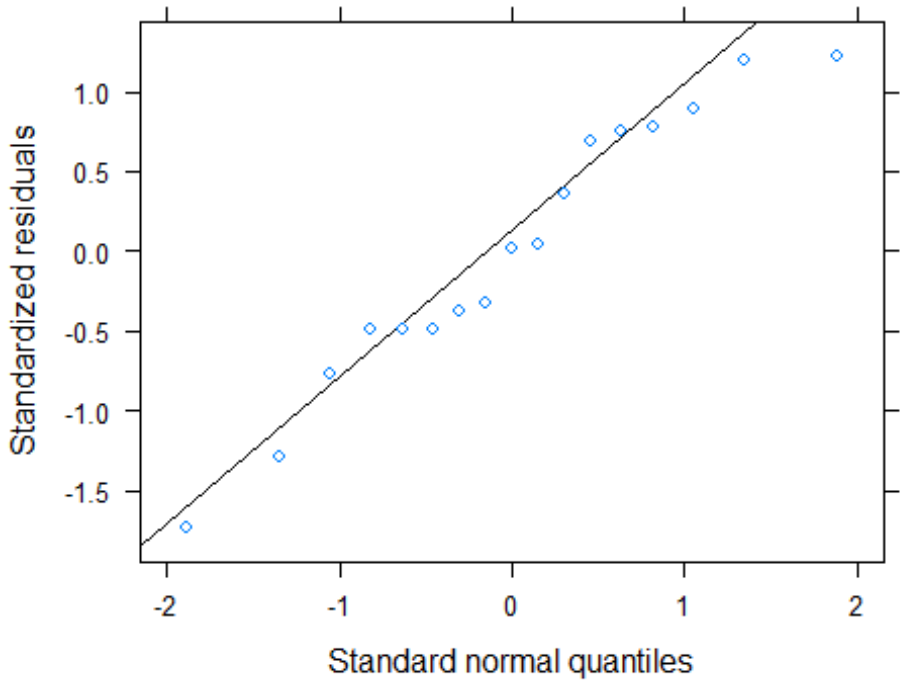
```
confint.merMod(STAGEAMODELP79,level=0.95,method="Wald")
```

```
##                    2.5 %       97.5 %
## .sig01                NA           NA
## .sigma                NA           NA
## (Intercept) 12.883284002 16.892529061
## VAP          0.003492012  0.023897592
## SpermCount   0.001157256  0.006031355
## StatusS     -0.881320127 -0.097689886
```

```
plot(STAGEAMODELP79, results="hide", fig.show='hide')#Visual Check Variance a
ssumption
```




```
qqmath(STAGEAMODELP79)#Visual Check Normality assumption
```



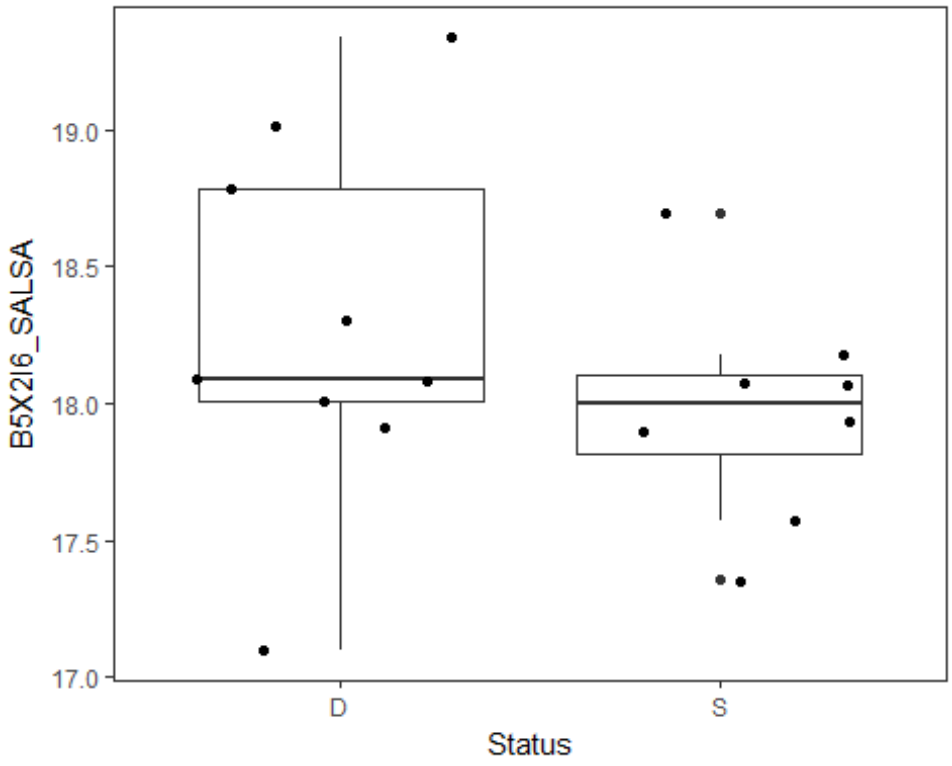

```
shapiro.test(resid(STAGEAMODELP79))#Test Check Normality assumption
```

```
##
##  Shapiro-Wilk normality test
##
## data:  resid(STAGEAMODELP79)
## W = 0.95356, p-value = 0.5151
```

```
rand(STAGEAMODELP79)#Test for significance of random predictor male ID
```

```
## Analysis of Random effects Table:
##      Chi.sq Chi.DF p.value
## Week  0.189      1     0.7
```

```
#Plot by status
ggplot(data=StageAonly, aes(x=Status, y=B5X2I6_SALSA)) +
  geom_boxplot()+ geom_jitter()+ theme_bw()+
  theme(panel.grid.minor=element_blank(),panel.grid.major=element_blank())
```




```
proteins[[100]]
```

```
## [1] "B5X4T0_SALSA"
```

```
STAGEAMODELP100<-lmer(B5X4T0_SALSA ~ rescale(VAP) + rescale(SpermCount) + Sta
tus +
                        + (1|Week),data=StageAonly)
summary(STAGEAMODELP100)
```

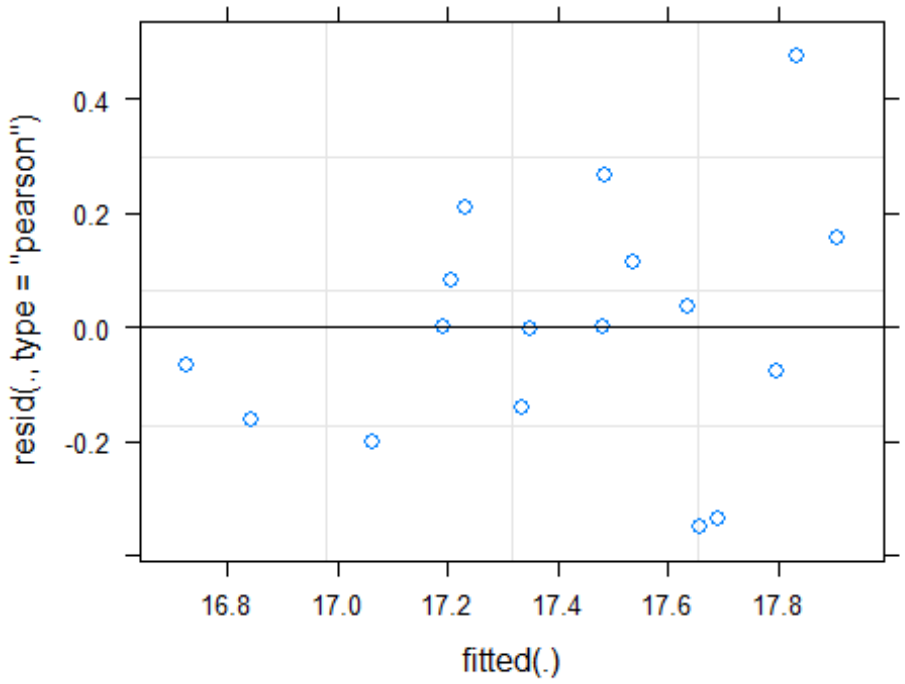
```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
```

```
## Formula: B5X4T0_SALSA ~ VAP + SpermCount + Status + +(1 | Week)
##    Data: StageAonly
##
## REML criterion at convergence: 37.6
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.26898 -0.51415 -0.00344  0.40683  1.71985
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  Week     (Intercept) 0.27202  0.5216
##  Residual             0.07674  0.2770
## Number of obs: 17, groups:  Week, 5
##
## Fixed effects:
##               Estimate Std. Error         df t value Pr(>|t|)
## (Intercept) 17.1115855  0.8336478 10.8360000  20.526 5.08e-10 ***
## VAP          0.0002128  0.0041768  9.3310000   0.051    0.960
## SpermCount   0.0012670  0.0009343  8.9830000   1.356    0.208
## StatusS     -0.4285552  0.1502889  9.0320000  -2.852    0.019 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn
## VAP         -0.896
## SpermCount -0.463  0.157
## StatusS     0.152 -0.157 -0.275
```

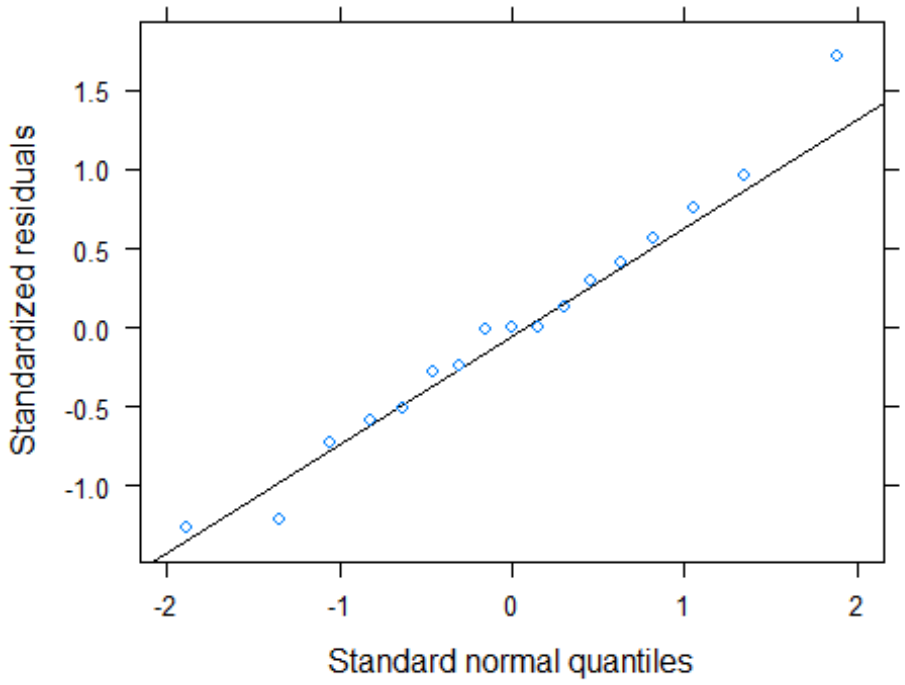
```
confint.merMod(STAGEAMODELP100,level=0.95,method="Wald")
```

```
##                     2.5 %       97.5 %
## .sig01                 NA           NA
## .sigma                 NA           NA
## (Intercept) 15.4776659126 18.745505086
## VAP         -0.0079735910  0.008399272
## SpermCount  -0.0005641601  0.003098087
## StatusS     -0.7231159532 -0.133994368
```

```
plot(STAGEAMODELP100, results="hide", fig.show='hide')#Visual Check Variance
assumption
```




```
qqmath(STAGEAMODELP100)#Visual Check Normality assumption
```



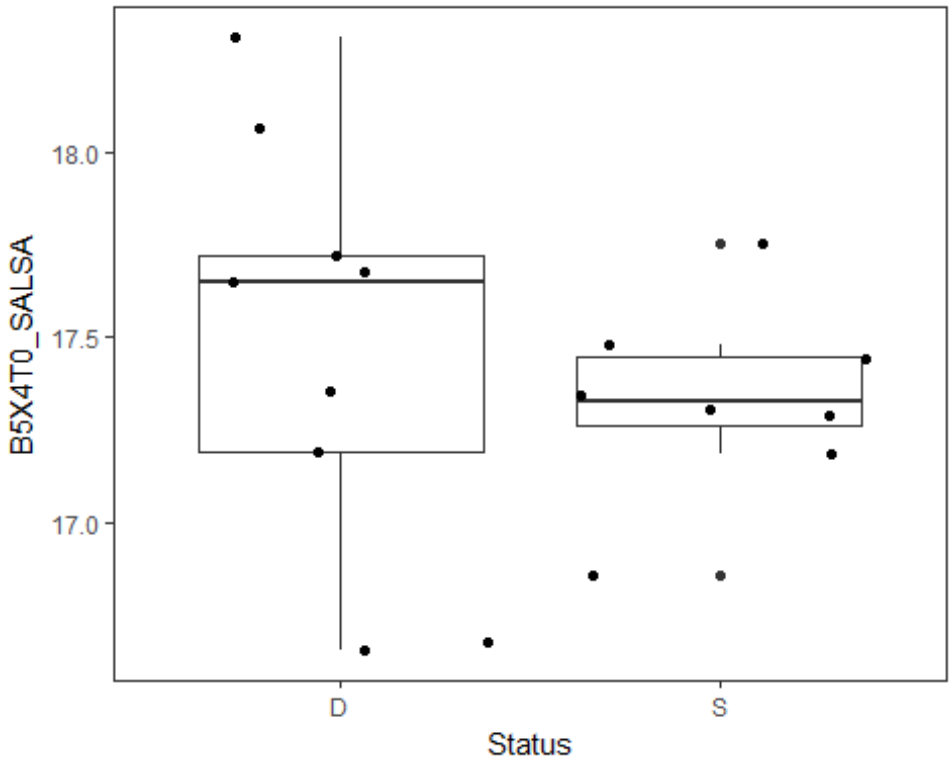

```
shapiro.test(resid(STAGEAMODELP100))#Test Check Normality assumption
```

```
##
##  Shapiro-Wilk normality test
##
## data:  resid(STAGEAMODELP100)
## W = 0.97995, p-value = 0.9567
```

```
rand(STAGEAMODELP100)#Test for significance of random predictor male ID
```

```
## Analysis of Random effects Table:
##      Chi.sq Chi.DF p.value
## Week   4.86      1    0.03 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
#Plot by status
ggplot(data=StageAonly, aes(x=Status, y=B5X4T0_SALSA)) +
  geom_boxplot()+ geom_jitter()+ theme_bw()+
  theme(panel.grid.minor=element_blank(),panel.grid.major=element_blank())
```




```
proteins[[141]]
```

```
## [1] "B5X9Z8_SALSA"
```

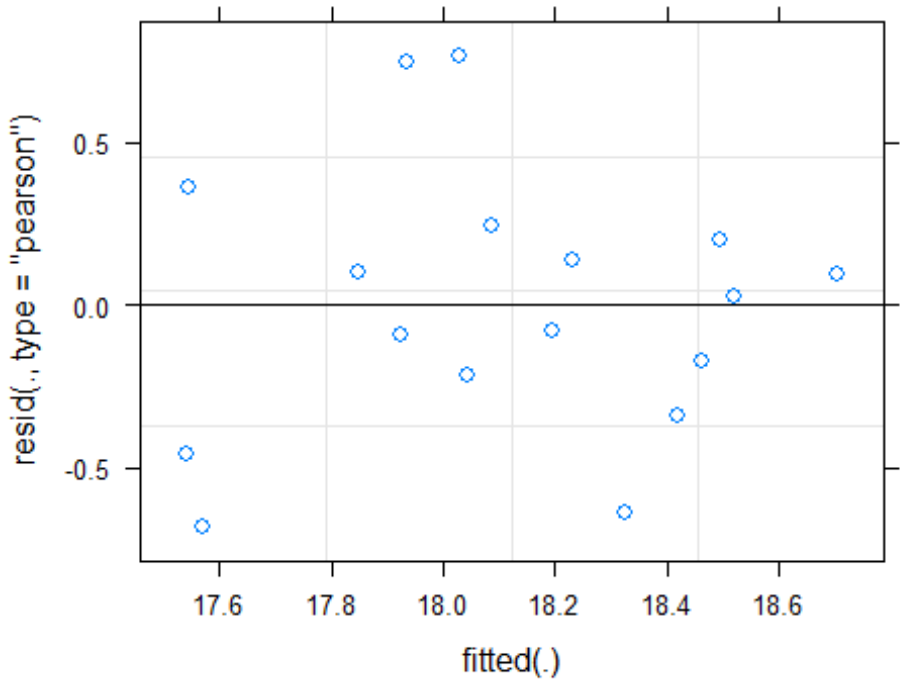
```
STAGEAMODELP141<-lmer(B5X9Z8_SALSA ~ rescale(VAP) + rescale(SpermCount) + Sta
tus +
                        + (1|Week),data=StageAonly)
summary(STAGEAMODELP141)
```

```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: B5X9Z8_SALSA ~ VAP + SpermCount + Status + +(1 | Week)
##    Data: StageAonly
##
## REML criterion at convergence: 42
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.47519 -0.47055  0.05069  0.43560  1.65668
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  Week     (Intercept) 0.008443 0.09188
##  Residual             0.214606 0.46326
## Number of obs: 17, groups:  Week, 5
##
## Fixed effects:
##              Estimate Std. Error        df t value Pr(>|t|)
## (Intercept) 17.598225   1.160035 12.931000  15.170 1.29e-09 ***
## VAP          0.007238   0.005862 12.546000   1.235    0.240
## SpermCount  -0.001498   0.001441 12.247000  -1.040    0.318
## StatusS     -0.568650   0.232441 11.842000  -2.446    0.031 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn
## VAP         -0.920
## SpermCount -0.524  0.178
## StatusS     0.123 -0.171 -0.179
```

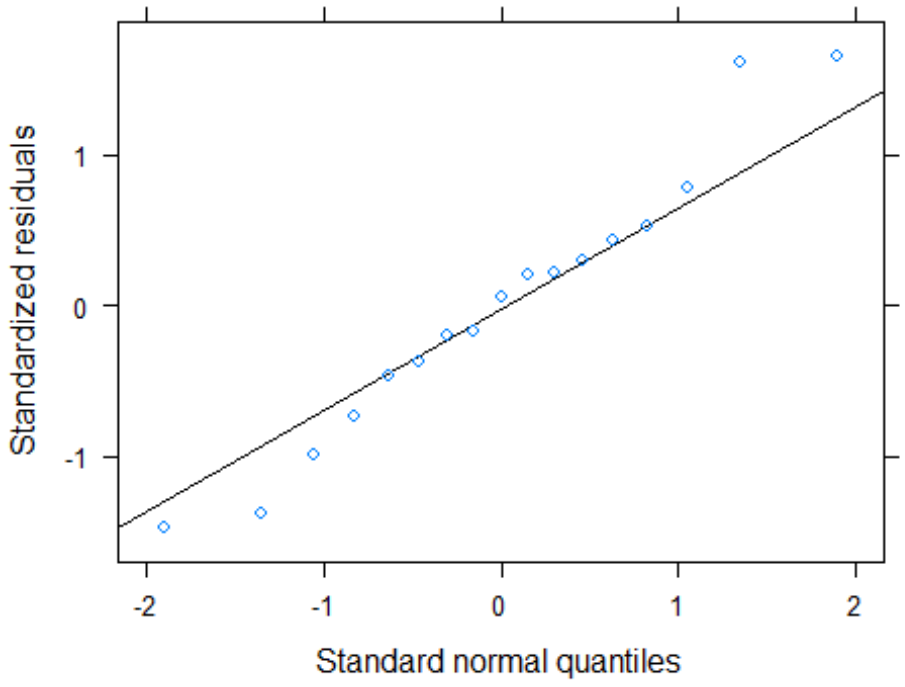
```
confint.merMod(STAGEAMODELP141,level=0.95,method="Wald")
```

```
##                    2.5 %       97.5 %
## .sig01                NA           NA
## .sigma                NA           NA
## (Intercept) 15.324598032 19.871850973
## VAP         -0.004250306  0.018726525
## SpermCount  -0.004322485  0.001325553
## StatusS     -1.024226201 -0.113073974
```

```
plot(STAGEAMODELP141, results="hide", fig.show='hide')#Visual Check Variance
assumption
```




```
qqmath(STAGEAMODELP141)#Visual Check Normality assumption
```



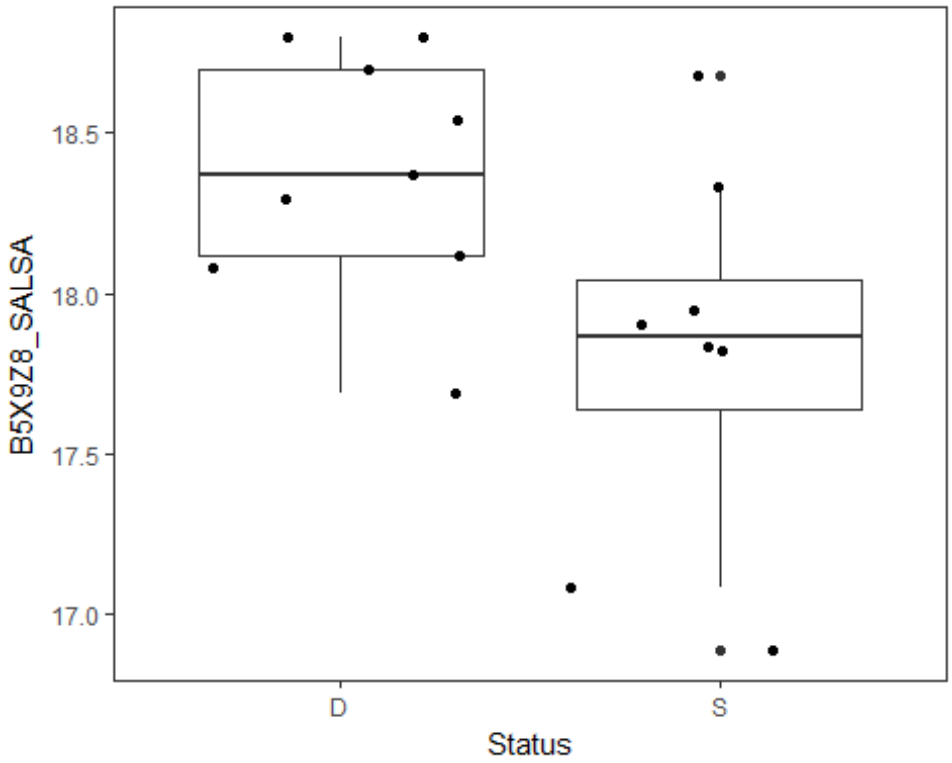

```
shapiro.test(resid(STAGEAMODELP141))#Test Check Normality assumption
```

```
##
##  Shapiro-Wilk normality test
##
## data:  resid(STAGEAMODELP141)
## W = 0.96497, p-value = 0.7258
```

```
rand(STAGEAMODELP141)#Test for significance of random predictor male ID
```

```
## Analysis of Random effects Table:
##      Chi.sq Chi.DF p.value
## Week 0.0228      1     0.9
```

```
#Plot by status
ggplot(data=StageAonly, aes(x=Status, y=B5X9Z8_SALSA)) +
  geom_boxplot()+ geom_jitter()+ theme_bw()+
  theme(panel.grid.minor=element_blank(),panel.grid.major=element_blank())
```




```
proteins[[146]]
```

```
## [1] "B5XAP1_SALSA"
```

```
STAGEAMODELP146<-lmer(B5XAP1_SALSA ~ rescale(VAP) + rescale(SpermCount) + Sta
tus +
                        + (1|Week),data=StageAonly)
summary(STAGEAMODELP146)
```

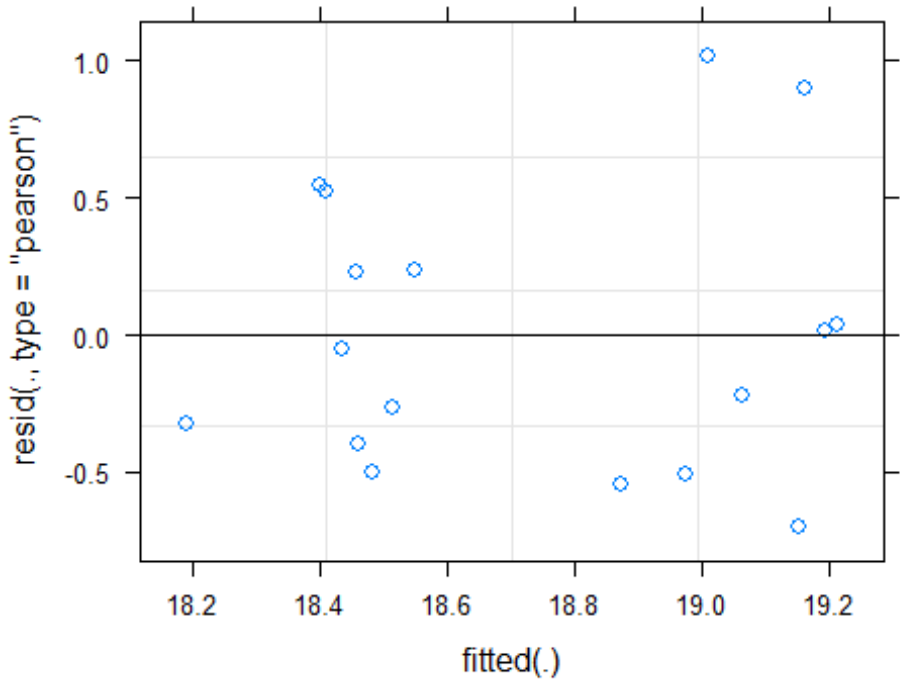
```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
```

```
## Formula: B5XAP1_SALSA ~ VAP + SpermCount + Status + +(1 | Week)
##    Data: StageAonly
##
## REML criterion at convergence: 47.8
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.2138 -0.6886 -0.0839  0.4035  1.7634
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  Week     (Intercept) 0.01508  0.1228
##  Residual             0.33500  0.5788
## Number of obs: 17, groups:  Week, 5
##
## Fixed effects:
##               Estimate Std. Error         df t value Pr(>|t|)
## (Intercept) 18.1137005  1.4528964 12.9220000  12.467 1.41e-08 ***
## VAP          0.0032980  0.0073447 12.4190000   0.449   0.6611
## SpermCount  -0.0007851  0.0018023 11.8910000  -0.436   0.6709
## StatusS      0.6555202  0.2906852 11.3550000   2.255   0.0448 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn
## VAP         -0.921
## SpermCount -0.523  0.178
## StatusS     0.124 -0.171 -0.180
```

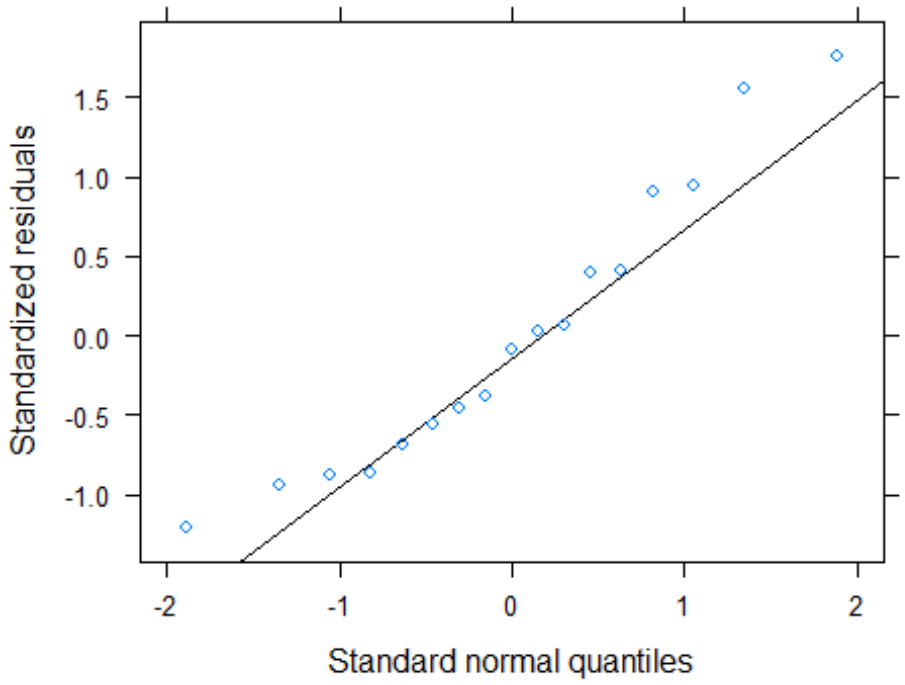
```
confint.merMod(STAGEAMODELP146,level=0.95,method="Wald")
```

```
##                    2.5 %       97.5 %
## .sig01                NA           NA
## .sigma                NA           NA
## (Intercept) 15.266075938 20.961325022
## VAP         -0.011097341  0.017693321
## SpermCount  -0.004317589  0.002747474
## StatusS      0.085787646  1.225252656
```

```
plot(STAGEAMODELP146, results="hide", fig.show='hide')#Visual Check Variance
assumption
```




```
qqmath(STAGEAMODELP146)#Visual Check Normality assumption
```



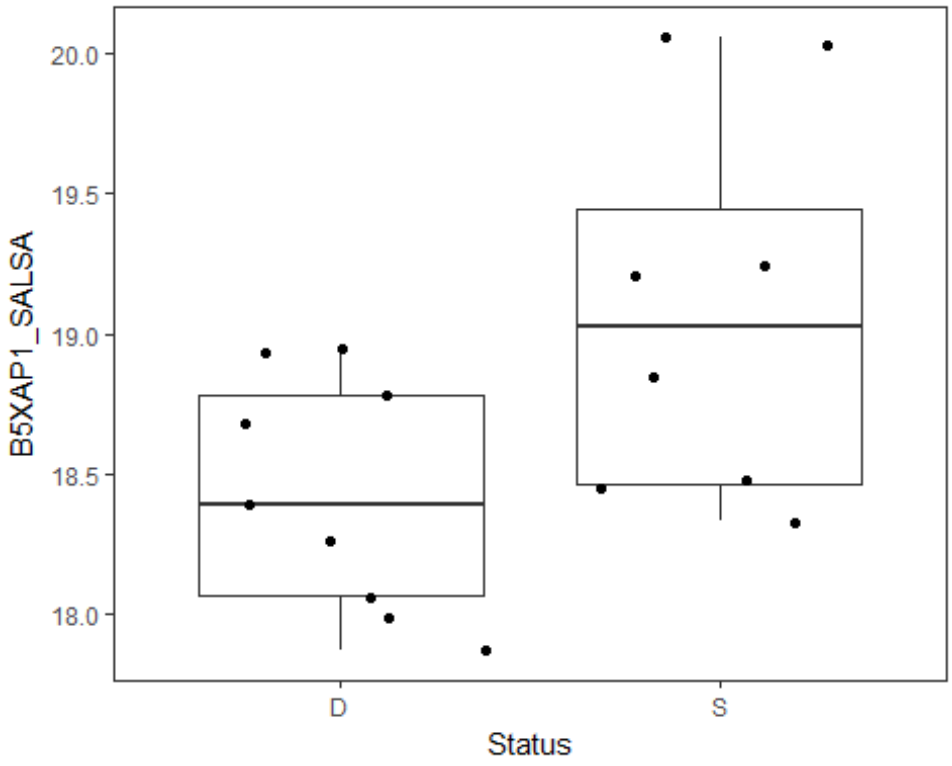

```
shapiro.test(resid(STAGEAMODELP146))#Test Check Normality assumption
```

```
##
##  Shapiro-Wilk normality test
##
## data:  resid(STAGEAMODELP146)
## W = 0.93763, p-value = 0.2912
```

```
rand(STAGEAMODELP146)#Test for significance of random predictor male ID
```

```
## Analysis of Random effects Table:
##      Chi.sq Chi.DF p.value
## Week 0.0198      1     0.9
```

```
#Plot by status
ggplot(data=StageAonly, aes(x=Status, y=B5XAP1_SALSA)) +
  geom_boxplot()+ geom_jitter()+ theme_bw()+
  theme(panel.grid.minor=element_blank(),panel.grid.major=element_blank())
```




```
proteins[[198]]
```

```
## [1] "B8R4G1_ONCTS"
```

```
STAGEAMODELP198<-lmer(B8R4G1_ONCTS ~ rescale(VAP) + rescale(SpermCount) + Sta
tus +
         + (1|Week),data=StageAonly)
summary(STAGEAMODELP198)
```

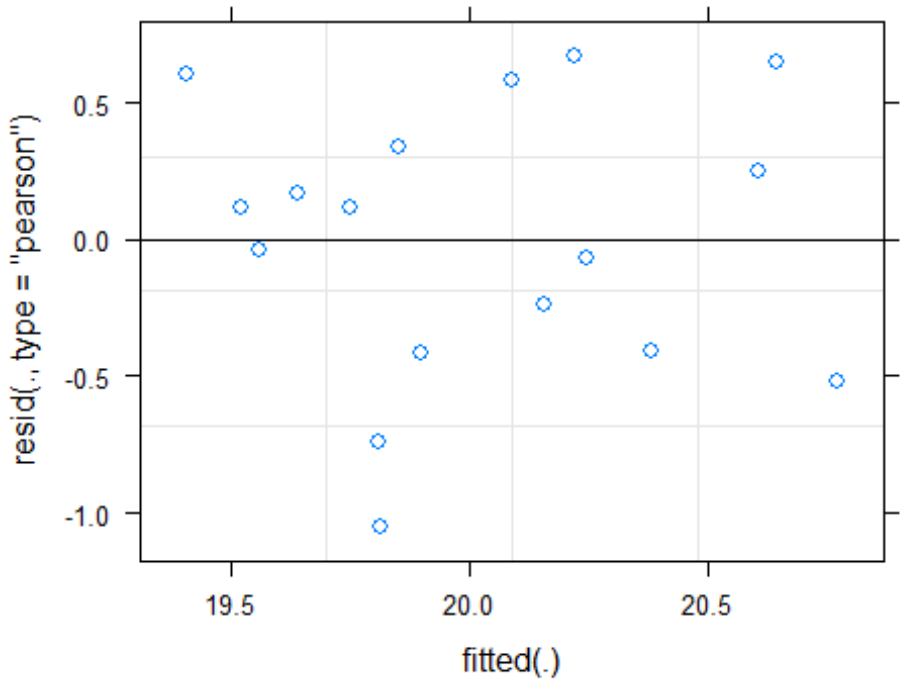
```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
```

```
## Formula: B8R4G1_ONCTS ~ VAP + SpermCount + Status + +(1 | Week)
##    Data: StageAonly
##
## REML criterion at convergence: 46.7
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.8667 -0.7210  0.2002  0.5986  1.1931
##
## Random effects:
##  Groups   Name        Variance  Std.Dev.
##  Week     (Intercept) 0.0005144 0.02268
##  Residual             0.3185817 0.56443
## Number of obs: 17, groups:  Week, 5
##
## Fixed effects:
##              Estimate Std. Error        df t value Pr(>|t|)
## (Intercept) 18.415941   1.387689 12.380000  13.271 1.09e-08 ***
## VAP          0.005019   0.006987 10.875000   0.718   0.4877
## SpermCount   0.003443   0.001740 11.620000   1.979   0.0720 .
## StatusS     -0.692078   0.281276 10.568000  -2.460   0.0325 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn
## VAP         -0.919
## SpermCount -0.529  0.180
## StatusS     0.117 -0.169 -0.170
```

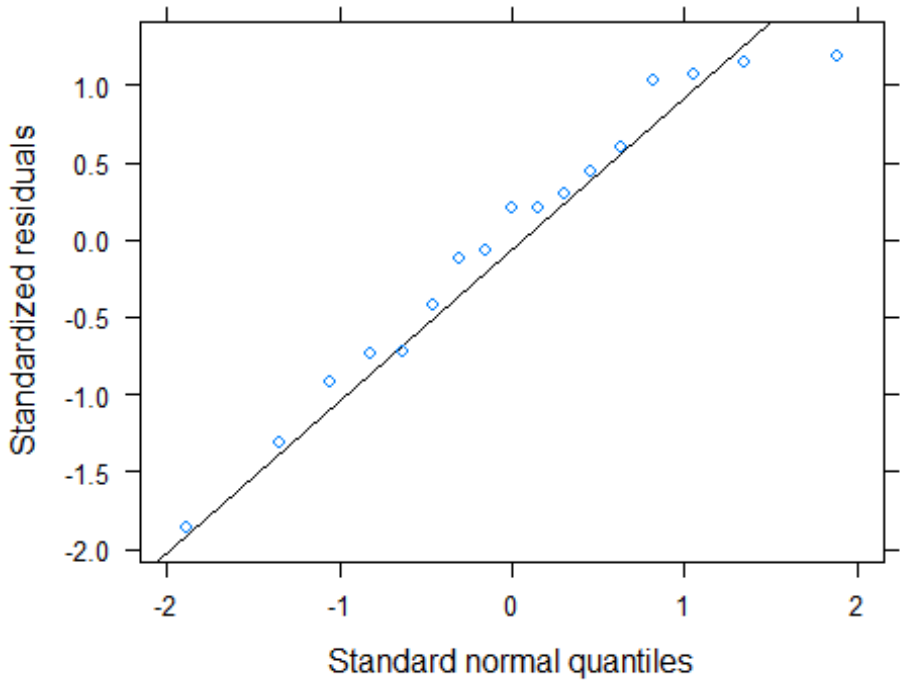
```
confint.merMod(STAGEAMODELP198,level=0.95,method="Wald")
```

```
##                     2.5 %       97.5 %
## .sig01                 NA           NA
## .sigma                 NA           NA
## (Intercept)  1.569612e+01 21.135761494
## VAP         -8.675637e-03  0.018713417
## SpermCount   3.298171e-05  0.006853502
## StatusS     -1.243369e+00 -0.140787654
```

```
plot(STAGEAMODELP198, results="hide", fig.show='hide')#Visual Check Variance
assumption
```




```
qqmath(STAGEAMODELP198)#Visual Check Normality assumption
```



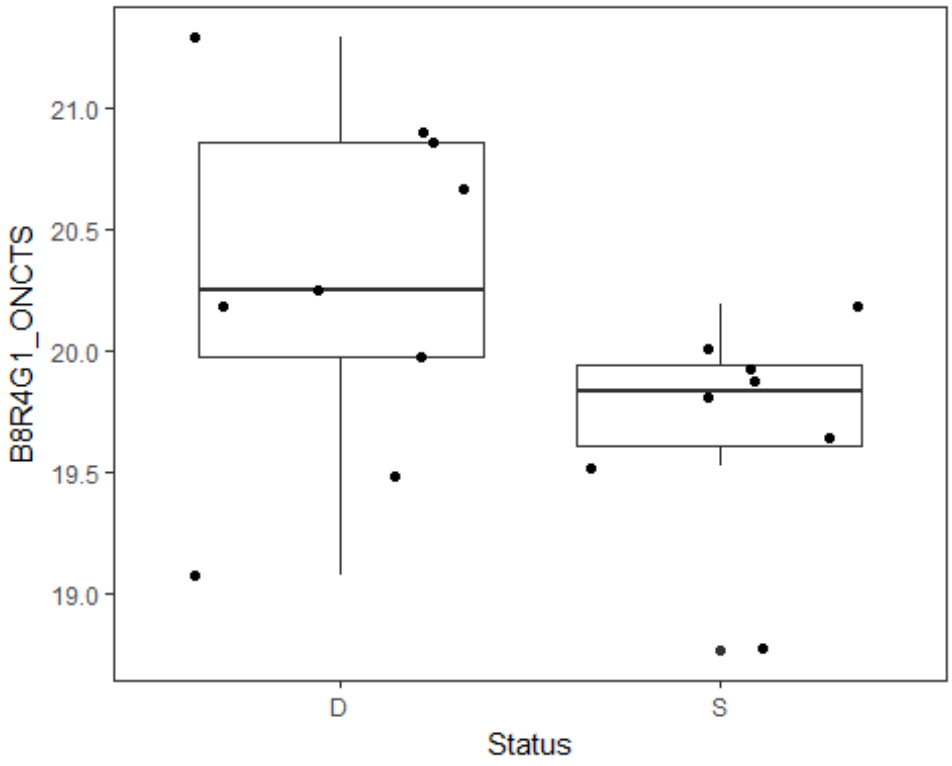

```
shapiro.test(resid(STAGEAMODELP198))#Test Check Normality assumption
```

```
##
##  Shapiro-Wilk normality test
##
## data:  resid(STAGEAMODELP198)
## W = 0.95413, p-value = 0.5251
```

```
rand(STAGEAMODELP198)#Test for significance of random predictor male ID
```

```
## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## Week 1.64e-05      1       1
```

```
#Plot by status
ggplot(data=StageAonly, aes(x=Status, y=B8R4G1_ONCTS)) +
geom_boxplot()+ geom_jitter()+ theme_bw()+
theme(panel.grid.minor=element_blank(),panel.grid.major=element_blank())
```




```
proteins[[199]]
```

```
## [1] "B8R4G2_ONCTS"
```

```
STAGEAMODELP199<-lmer(B8R4G2_ONCTS ~ rescale(VAP) + rescale(SpermCount) + Sta
tus +
         + (1|Week),data=StageAonly)
summary(STAGEAMODELP199)
```

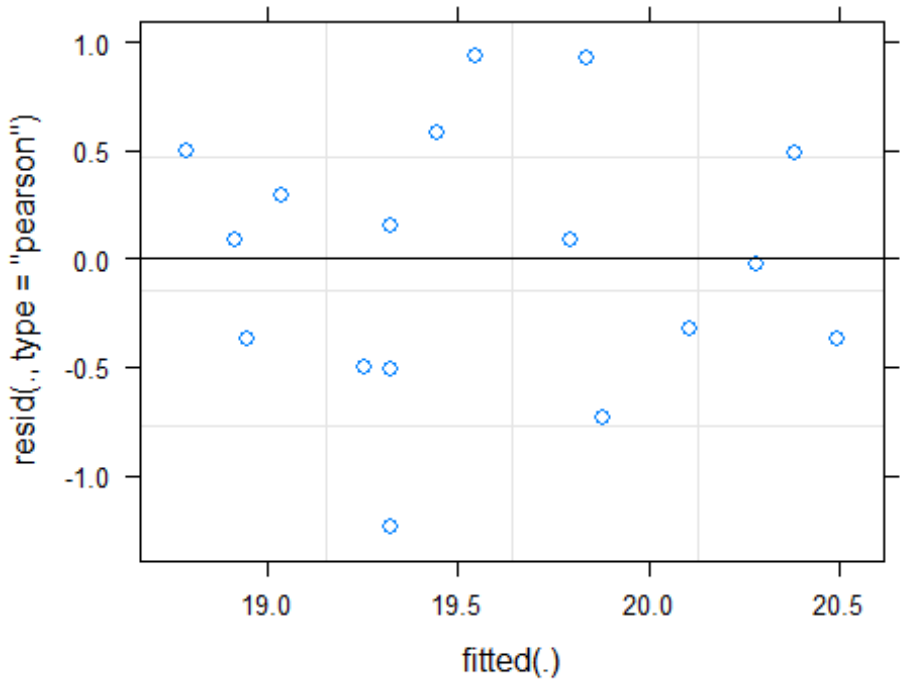
```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
```

```
## Formula: B8R4G2_ONCTS ~ VAP + SpermCount + Status + +(1 | Week)
##    Data: StageAonly
##
## REML criterion at convergence: 50.8
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.8812 -0.5568  0.1289  0.7457  1.4260
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  Week     (Intercept) 0.0000   0.0000
##  Residual             0.4357   0.6601
## Number of obs: 17, groups:  Week, 5
##
## Fixed effects:
##              Estimate Std. Error        df t value Pr(>|t|)
## (Intercept) 17.622784   1.621473 13.000000  10.868 6.79e-08 ***
## VAP          0.004692   0.008163 13.000000   0.575   0.5753
## SpermCount   0.004921   0.002034 13.000000   2.419   0.0309 *
## StatusS     -0.826348   0.328847 13.000000  -2.513   0.0260 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn
## VAP         -0.919
## SpermCount -0.529  0.180
## StatusS     0.117 -0.169 -0.169
```

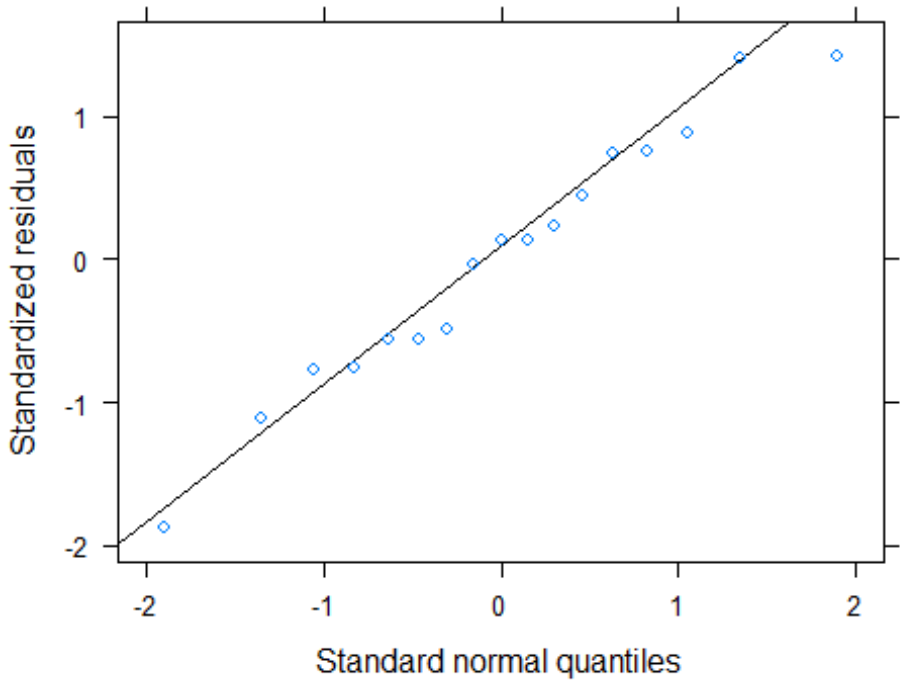
```
confint.merMod(STAGEAMODELP199,level=0.95,method="Wald")
```

```
##                     2.5 %       97.5 %
## .sig01                 NA           NA
## .sigma                 NA           NA
## (Intercept) 14.4447544155 20.800812709
## VAP         -0.0113073108  0.020690416
## SpermCount   0.0009345756  0.008907758
## StatusS     -1.4708755183 -0.181819997
```

```
plot(STAGEAMODELP199, results="hide", fig.show='hide')#Visual Check Variance
assumption
```




```
qqmath(STAGEAMODELP199)#Visual Check Normality assumption
```



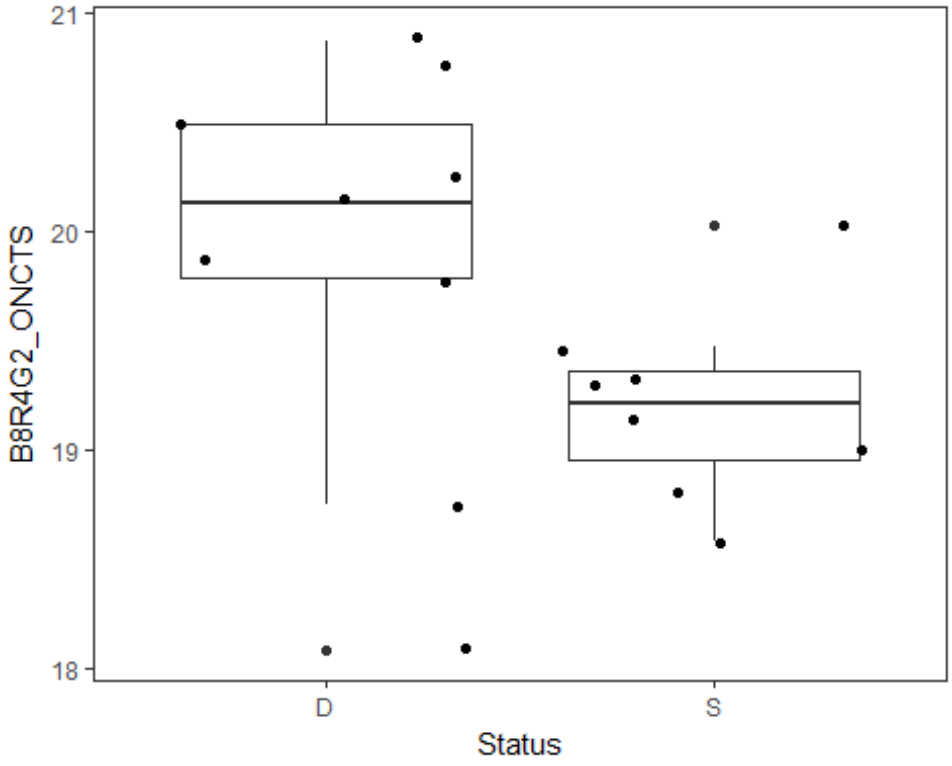

```
shapiro.test(resid(STAGEAMODELP199))#Test Check Normality assumption
```

```
##
##  Shapiro-Wilk normality test
##
## data:  resid(STAGEAMODELP199)
## W = 0.9724, p-value = 0.859
```

```
rand(STAGEAMODELP199)#Test for significance of random predictor male ID
```

```
## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## Week 3.55e-14      1       1
```

```
#Plot by status
ggplot(data=StageAonly, aes(x=Status, y=B8R4G2_ONCTS)) +
geom_boxplot()+ geom_jitter()+ theme_bw()+
theme(panel.grid.minor=element_blank(),panel.grid.major=element_blank())
```




```
proteins[[247]]
```

```
## [1] "C0HAB7_SALSA"
```

```
STAGEAMODELP247<-lmer(C0HAB7_SALSA ~ rescale(VAP) + rescale(SpermCount) + Sta
tus +
         + (1|Week),data=StageAonly)
summary(STAGEAMODELP247)
```

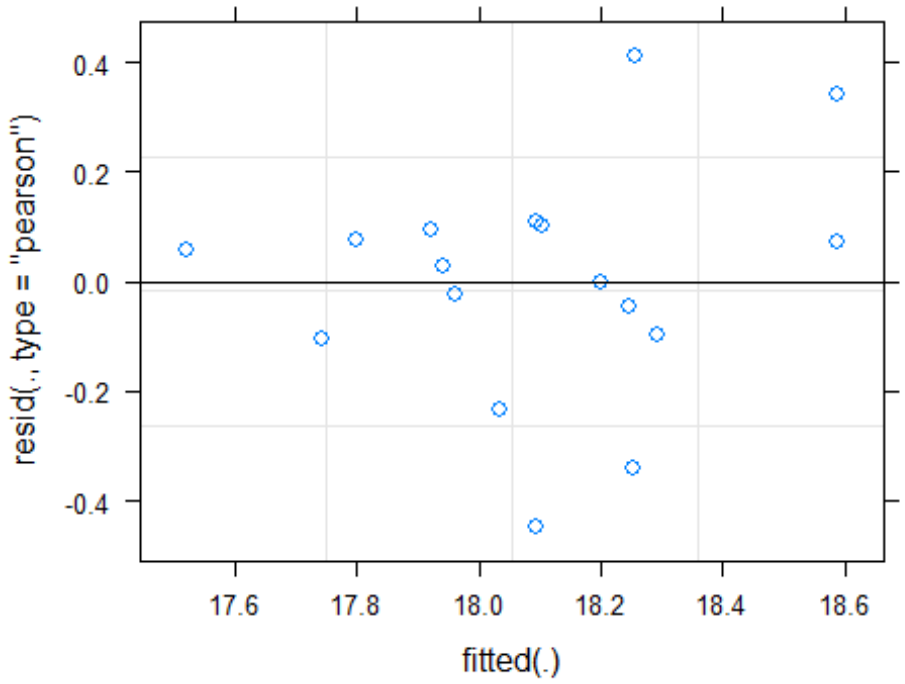
```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
```

```
## Formula: C0HAB7_SALSA ~ VAP + SpermCount + Status + +(1 | Week)
##    Data: StageAonly
##
## REML criterion at convergence: 30.7
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.7074 -0.3659  0.1061  0.3622  1.5717
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  Week     (Intercept) 0.04173  0.2043
##  Residual             0.06934  0.2633
## Number of obs: 17, groups:  Week, 5
##
## Fixed effects:
##               Estimate Std. Error         df t value Pr(>|t|)
## (Intercept) 17.9940786  0.7303476 11.9790000  24.638 1.25e-11 ***
## VAP          0.0036847  0.0037412 12.0460000   0.985   0.3440
## SpermCount  -0.0010291  0.0008617 10.8510000  -1.194   0.2578
## StatusS     -0.4174178  0.1383316 10.8660000  -3.018   0.0119 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn
## VAP         -0.922
## SpermCount -0.494  0.167
## StatusS     0.149 -0.168 -0.237
```

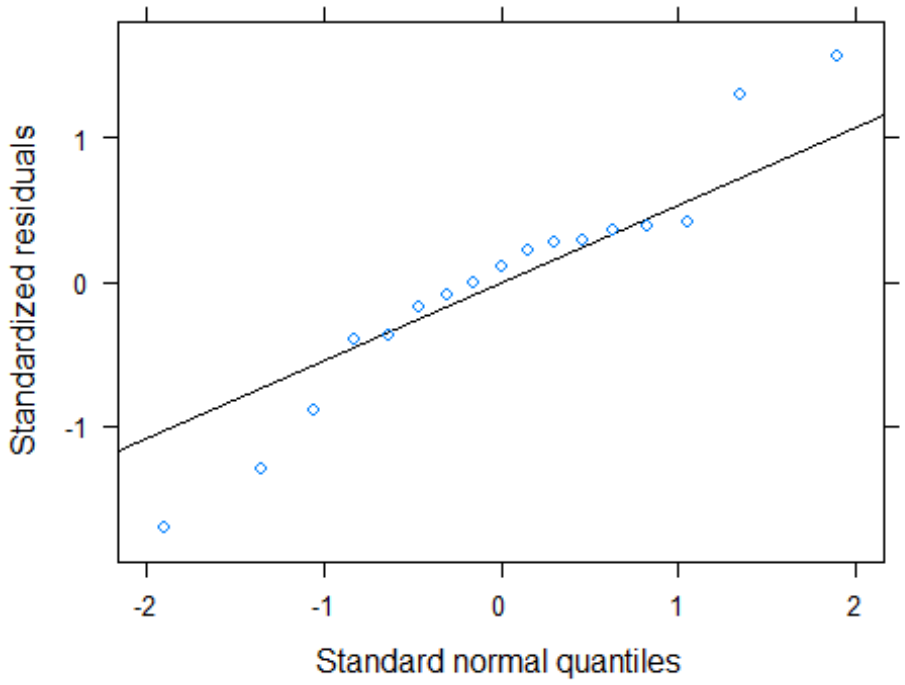
```
confint.merMod(STAGEAMODELP247,level=0.95,method="Wald")
```

```
##                    2.5 %        97.5 %
## .sig01                NA            NA
## .sigma                NA            NA
## (Intercept) 16.562623490 19.4255336241
## VAP         -0.003647956  0.0110172985
## SpermCount  -0.002718036  0.0006597913
## StatusS     -0.688542722 -0.1462929046
```

```
plot(STAGEAMODELP247, results="hide", fig.show='hide')#Visual Check Variance
assumption
```




```
qqmath(STAGEAMODELP247)#Visual Check Normality assumption
```



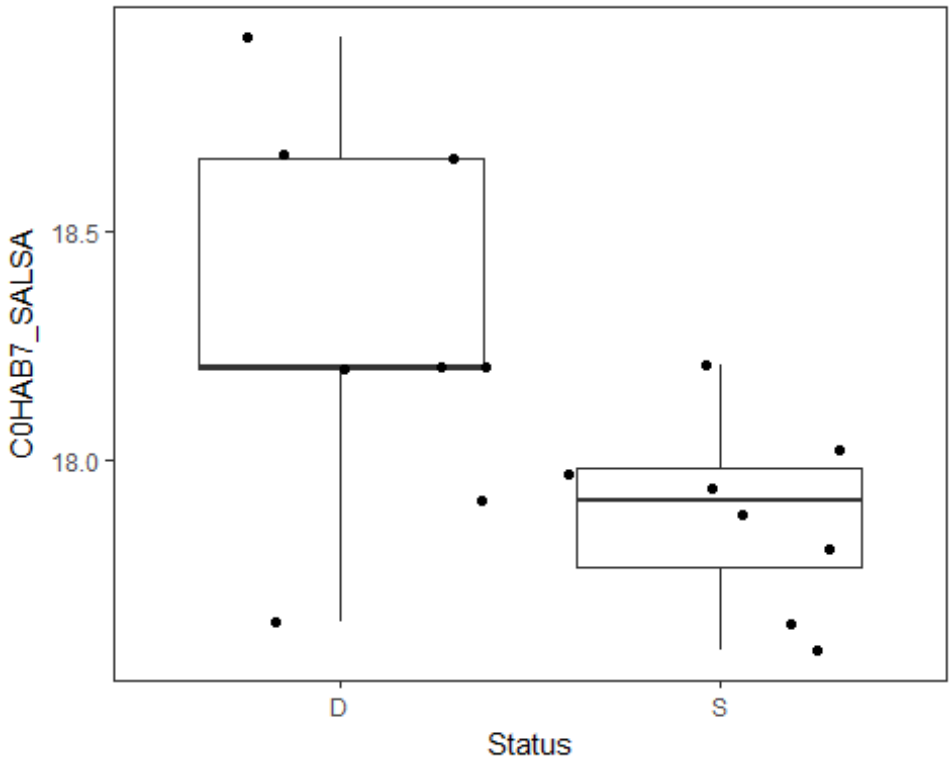

```
shapiro.test(resid(STAGEAMODELP247))#Test Check Normality assumption
```

```
##
##  Shapiro-Wilk normality test
##
## data:  resid(STAGEAMODELP247)
## W = 0.94899, p-value = 0.4407
```

```
rand(STAGEAMODELP247)#Test for significance of random predictor male ID
```

```
## Analysis of Random effects Table:
##      Chi.sq Chi.DF p.value
## Week   2.14      1     0.1
```

```
#Plot by status
ggplot(data=StageAonly, aes(x=Status, y=C0HAB7_SALSA)) +
geom_boxplot()+ geom_jitter()+ theme_bw()+
theme(panel.grid.minor=element_blank(),panel.grid.major=element_blank())
```




```
proteins[[248]]
```

```
## [1] "C0HAD5_SALSA"
```

```
STAGEAMODELP248<-lmer(C0HAD5_SALSA ~ rescale(VAP) + rescale(SpermCount) + Sta
tus +
         + (1|Week),data=StageAonly)
summary(STAGEAMODELP248)
```

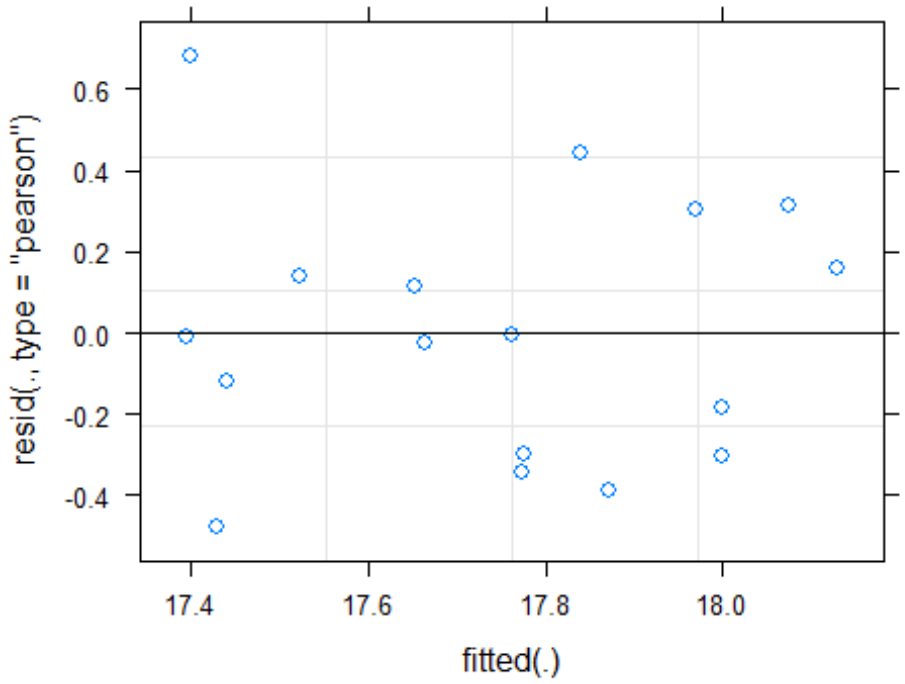
```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
```

```
## Formula: C0HAD5_SALSA ~ VAP + SpermCount + Status + +(1 | Week)
##    Data: StageAonly
##
## REML criterion at convergence: 34.6
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.35113 -0.83924 -0.03311  0.45011  1.92599
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  Week     (Intercept) 0.0000   0.0000
##  Residual             0.1262   0.3552
## Number of obs: 17, groups:  Week, 5
##
## Fixed effects:
##              Estimate Std. Error        df t value Pr(>|t|)
## (Intercept) 17.877664   0.872488 13.000000  20.490  2.8e-11 ***
## VAP         -0.002181   0.004392 13.000000  -0.496   0.6278
## SpermCount   0.001423   0.001094 13.000000   1.301   0.2160
## StatusS     -0.420792   0.176947 13.000000  -2.378   0.0334 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn
## VAP         -0.919
## SpermCount -0.529  0.180
## StatusS     0.117 -0.169 -0.169
```

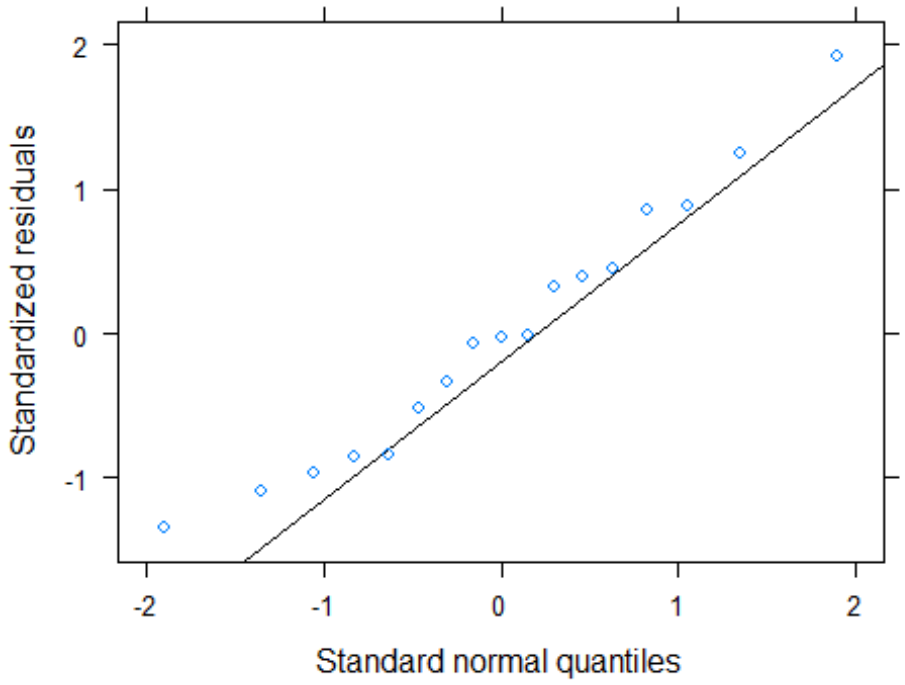
```
confint.merMod(STAGEAMODELP248,level=0.95,method="Wald")
```

```
##                     2.5 %       97.5 %
## .sig01                 NA           NA
## .sigma                 NA           NA
## (Intercept) 16.1676197203 19.587709070
## VAP         -0.0107894779  0.006427967
## SpermCount  -0.0007217021  0.003568535
## StatusS     -0.7676020806 -0.073982725
```

```
plot(STAGEAMODELP248, results="hide", fig.show='hide')#Visual Check Variance
assumption
```




```
qqmath(STAGEAMODELP248)#Visual Check Normality assumption
```



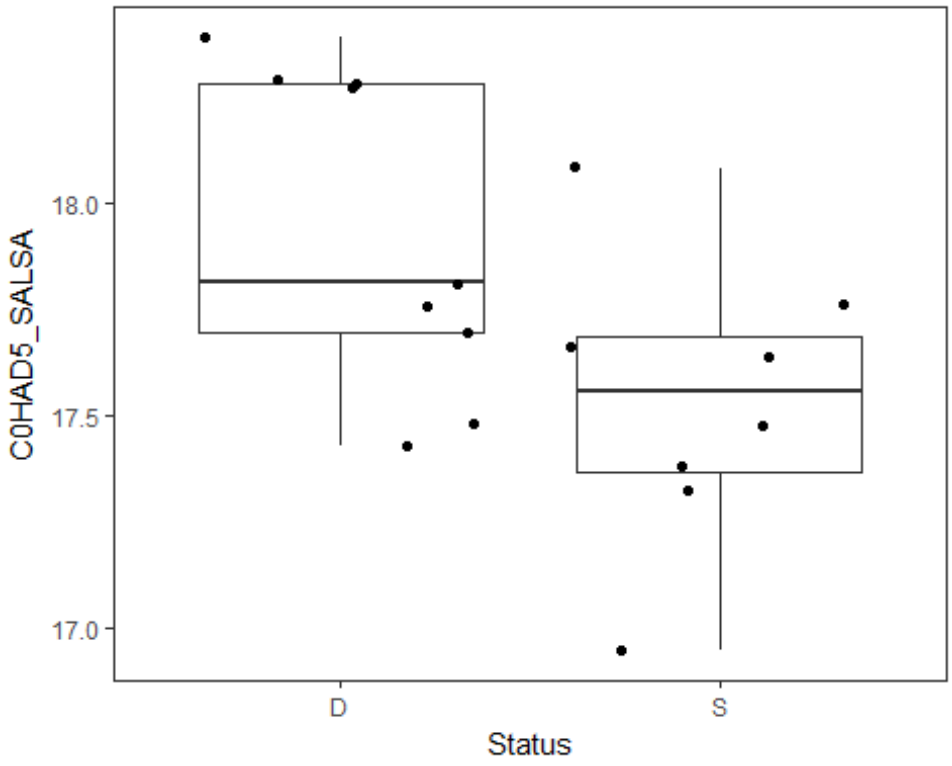

```
shapiro.test(resid(STAGEAMODELP248))#Test Check Normality assumption
```

```
##
##  Shapiro-Wilk normality test
##
## data:  resid(STAGEAMODELP248)
## W = 0.969, p-value = 0.8007
```

```
rand(STAGEAMODELP248)#Test for significance of random predictor male ID
```

```
## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## Week 4.26e-14      1       1
```

```
#Plot by status
ggplot(data=StageAonly, aes(x=Status, y=C0HAD5_SALSA)) +
geom_boxplot()+ geom_jitter()+ theme_bw()+
theme(panel.grid.minor=element_blank(),panel.grid.major=element_blank())
```




```
proteins[[262]]
```

```
## [1] "C0PUT9_SALSA"
```

```
STAGEAMODELP262<-lmer(C0PUT9_SALSA ~ rescale(VAP) + rescale(SpermCount) + Sta
tus +
         + (1|Week),data=StageAonly)
summary(STAGEAMODELP262)
```

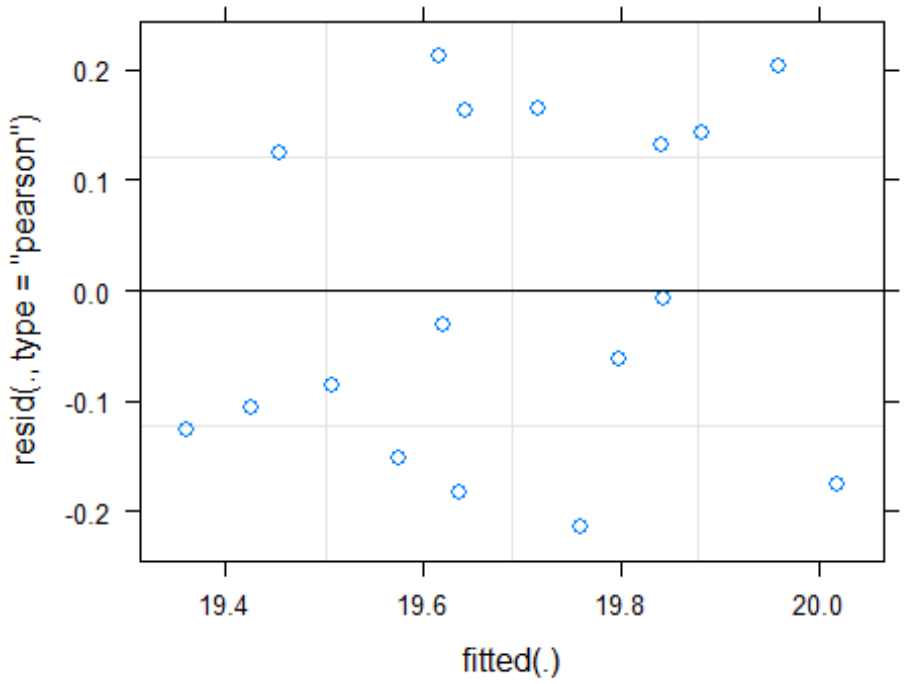
```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
```

```
## Formula: C0PUT9_SALSA ~ VAP + SpermCount + Status + +(1 | Week)
##    Data: StageAonly
##
## REML criterion at convergence: 22
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.1488 -0.6785 -0.1637  0.7635  1.1397
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  Week     (Intercept) 0.02218  0.1489
##  Residual             0.03490  0.1868
## Number of obs: 17, groups:  Week, 5
##
## Fixed effects:
##               Estimate Std. Error         df t value Pr(>|t|)
## (Intercept)  1.969e+01  5.195e-01  1.176e+01  37.900 1.17e-13 ***
## VAP          3.604e-05  2.661e-03  1.181e+01   0.014  0.98942
## SpermCount   4.791e-04  6.121e-04  1.050e+01   0.783  0.45108
## StatusS     -3.351e-01  9.827e-02  1.053e+01  -3.410  0.00619 **
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn
## VAP         -0.922
## SpermCount -0.494  0.167
## StatusS     0.149 -0.168 -0.239
```

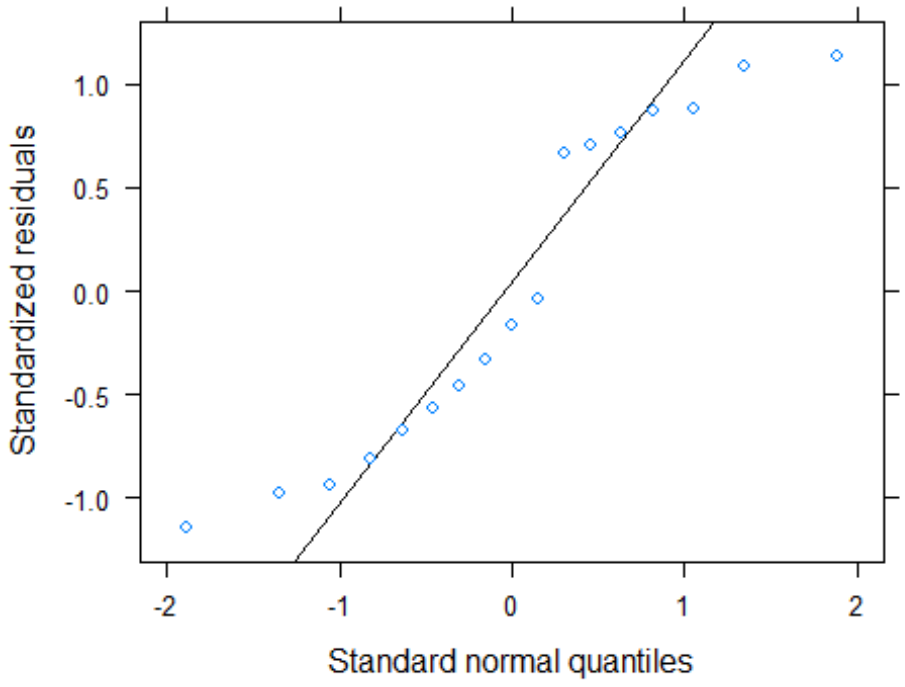
```
confint.merMod(STAGEAMODELP262,level=0.95,method="Wald")
```

```
##                      2.5 %       97.5 %
## .sig01                  NA           NA
## .sigma                  NA           NA
## (Intercept) 18.6708300987 20.707223947
## VAP         -0.0051800764  0.005252155
## SpermCount  -0.0007206099  0.001678854
## StatusS     -0.5276906061 -0.142492152
```

```
plot(STAGEAMODELP262, results="hide", fig.show='hide')#Visual Check Variance
assumption
```




```
qqmath(STAGEAMODELP262)#Visual Check Normality assumption
```



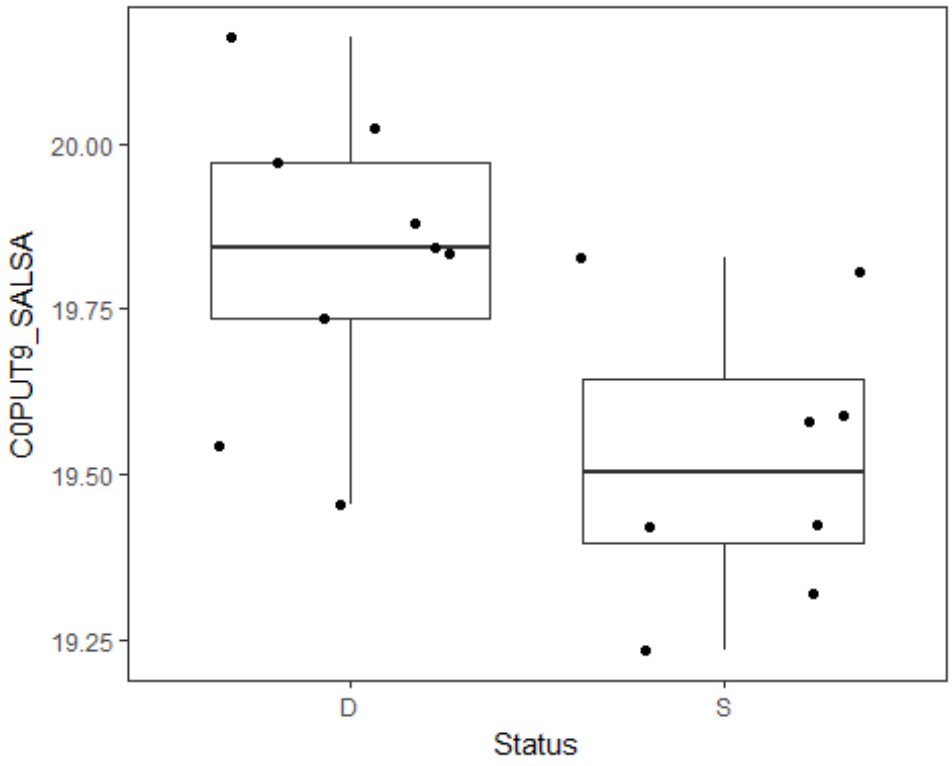

```
shapiro.test(resid(STAGEAMODELP262))#Test Check Normality assumption
```

```
##
##  Shapiro-Wilk normality test
##
## data:  resid(STAGEAMODELP262)
## W = 0.8939, p-value = 0.05375
```

```
rand(STAGEAMODELP262)#Test for significance of random predictor male ID
```

```
## Analysis of Random effects Table:
##      Chi.sq Chi.DF p.value
## Week   1.78      1     0.2
```

```
#Plot by status
ggplot(data=StageAonly, aes(x=Status, y=C0PUT9_SALSA)) +
geom_boxplot()+ geom_jitter()+ theme_bw()+
theme(panel.grid.minor=element_blank(),panel.grid.major=element_blank())
```




```
proteins[[277]]
```

```
## [1] "C1BFZ2_ONCMY"
```

```
STAGEAMODELP277<-lmer(C1BFZ2_ONCMY ~ rescale(VAP) + rescale(SpermCount) + Sta
tus +
         + (1|Week),data=StageAonly)
summary(STAGEAMODELP277)
```

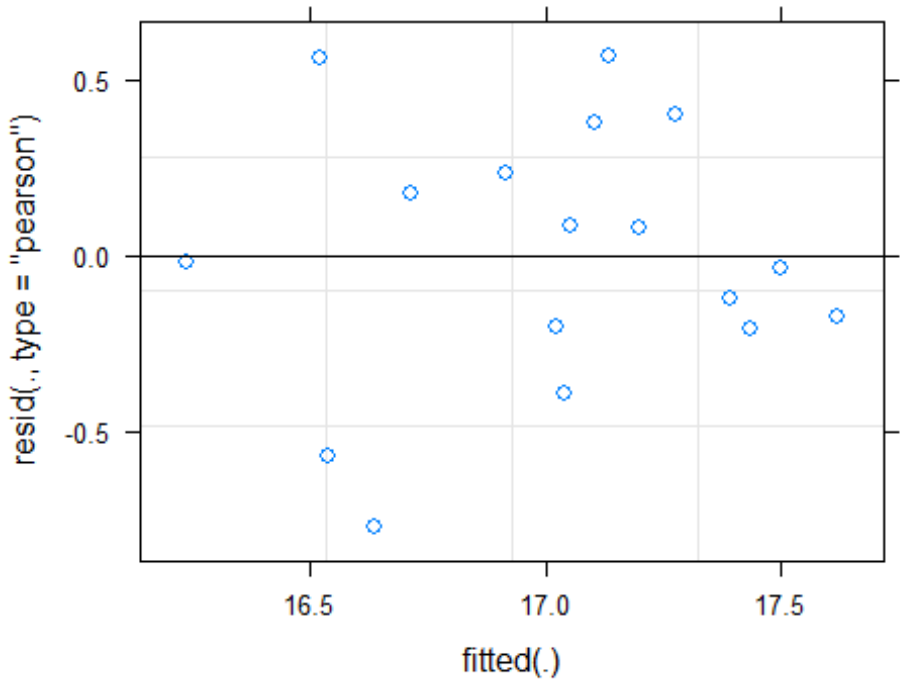
```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
```

```
## Formula: C1BFZ2_ONCMY ~ VAP + SpermCount + Status + +(1 | Week)
##    Data: StageAonly
##
## REML criterion at convergence: 40.9
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.78797 -0.46134 -0.04775  0.53936  1.31948
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  Week     (Intercept) 0.02184  0.1478
##  Residual             0.18710  0.4326
## Number of obs: 17, groups:  Week, 5
##
## Fixed effects:
##              Estimate Std. Error        df t value Pr(>|t|)
## (Intercept) 19.173750   1.114125 12.987000  17.210 2.54e-10 ***
## VAP         -0.012082   0.005658 12.948000  -2.135   0.0524 .
## SpermCount  -0.001188   0.001364 11.783000  -0.871   0.4014
## StatusS      0.632912   0.219513 11.486000   2.883   0.0143 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##            (Intr) VAP    SprmCn
## VAP        -0.922
## SpermCount -0.516  0.176
## StatusS     0.132 -0.172 -0.194
```

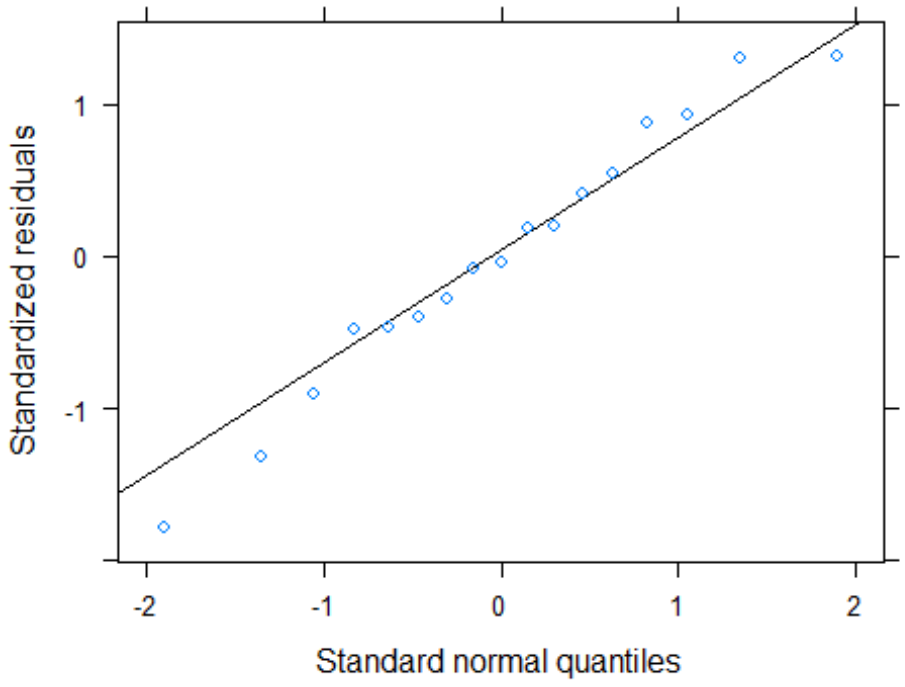
```
confint.merMod(STAGEAMODELP277,level=0.95,method="Wald")
```

```
##                    2.5 %        97.5 %
## .sig01                NA            NA
## .sigma                NA            NA
## (Intercept) 16.990105818 21.3573943789
## VAP         -0.023170520 -0.0009926367
## SpermCount  -0.003861153  0.0014860002
## StatusS      0.202674479  1.0631488638
```

```
plot(STAGEAMODELP277, results="hide", fig.show='hide')#Visual Check Variance
assumption
```




```
qqmath(STAGEAMODELP277)#Visual Check Normality assumption
```



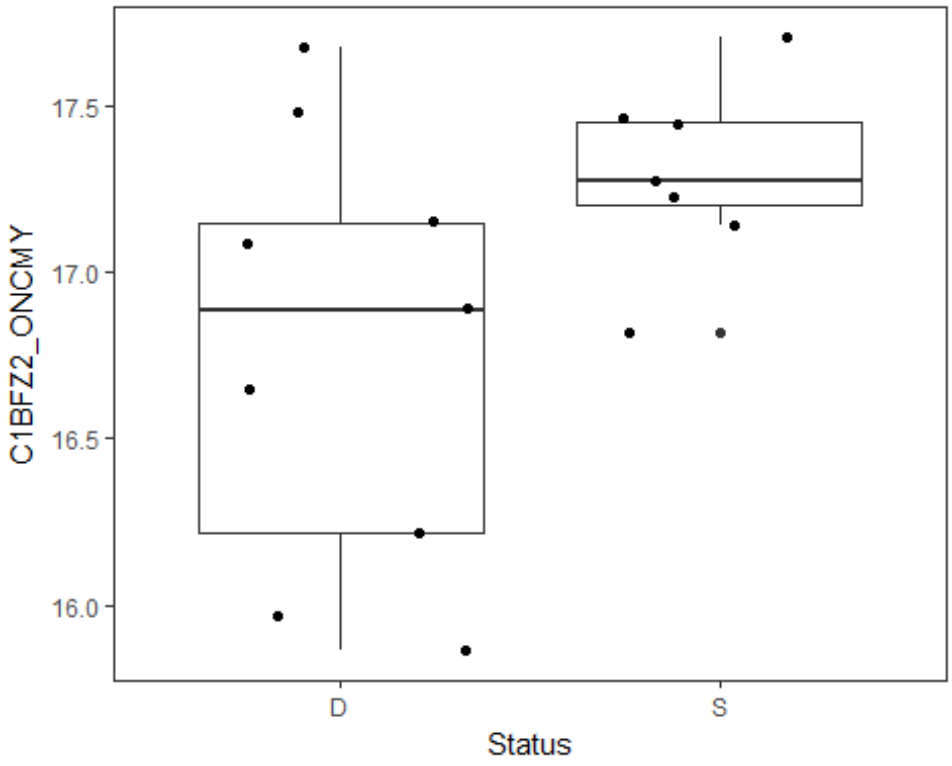

```
shapiro.test(resid(STAGEAMODELP277))#Test Check Normality assumption
```

```
##
##  Shapiro-Wilk normality test
##
## data:  resid(STAGEAMODELP277)
## W = 0.97352, p-value = 0.8767
```

```
rand(STAGEAMODELP277)#Test for significance of random predictor male ID
```

```
## Analysis of Random effects Table:
##      Chi.sq Chi.DF p.value
## Week  0.147      1     0.7
```

```
#Plot by status
ggplot(data=StageAonly, aes(x=Status, y=C1BFZ2_ONCMY)) +
geom_boxplot()+ geom_jitter()+ theme_bw()+
theme(panel.grid.minor=element_blank(),panel.grid.major=element_blank())
```




```
proteins[[305]]
```

```
## [1] "F8LFR3_ONCMY"
```

```
STAGEAMODELP305<-lmer(F8LFR3_ONCMY ~ rescale(VAP) + rescale(SpermCount) + Sta
tus +
         + (1|Week),data=StageAonly)
summary(STAGEAMODELP305)
```

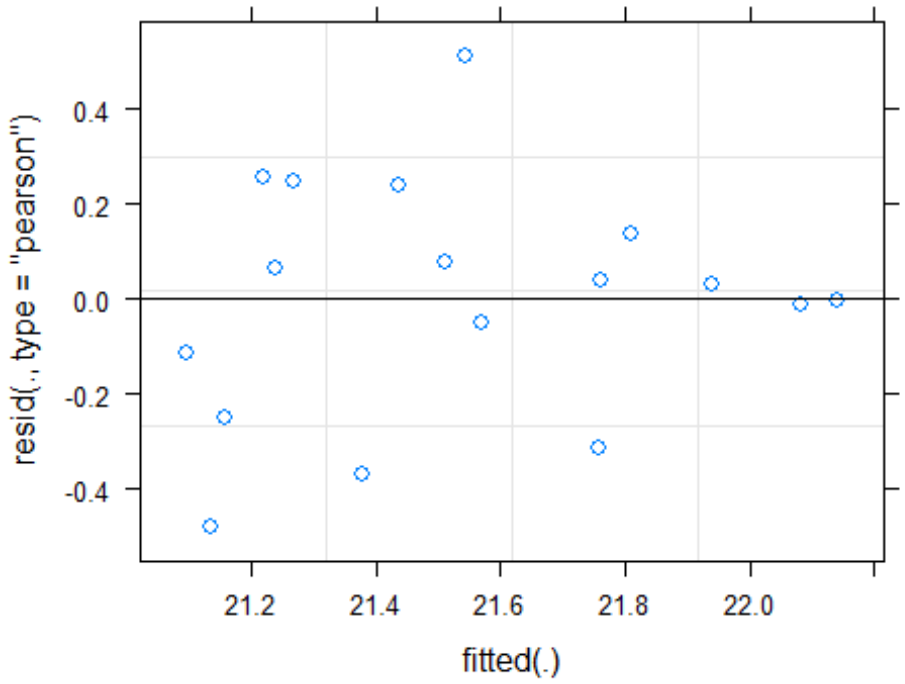
```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
```

```
## Formula: F8LFR3_ONCMY ~ VAP + SpermCount + Status + +(1 | Week)
##    Data: StageAonly
##
## REML criterion at convergence: 33.5
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.5778 -0.3773  0.0990  0.4540  1.6793
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  Week     (Intercept) 0.03499  0.1871
##  Residual             0.09319  0.3053
## Number of obs: 17, groups:  Week, 5
##
## Fixed effects:
##               Estimate Std. Error         df t value Pr(>|t|)
## (Intercept) 20.8230762  0.8274363 12.4330000  25.166 4.85e-12 ***
## VAP         -0.0001831  0.0042317 12.6060000  -0.043  0.96617
## SpermCount   0.0031165  0.0009875 11.2360000   3.156  0.00893 **
## StatusS     -0.4711875  0.1585514 11.1870000  -2.972  0.01249 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn
## VAP         -0.923
## SpermCount -0.502  0.170
## StatusS     0.145 -0.170 -0.223
```

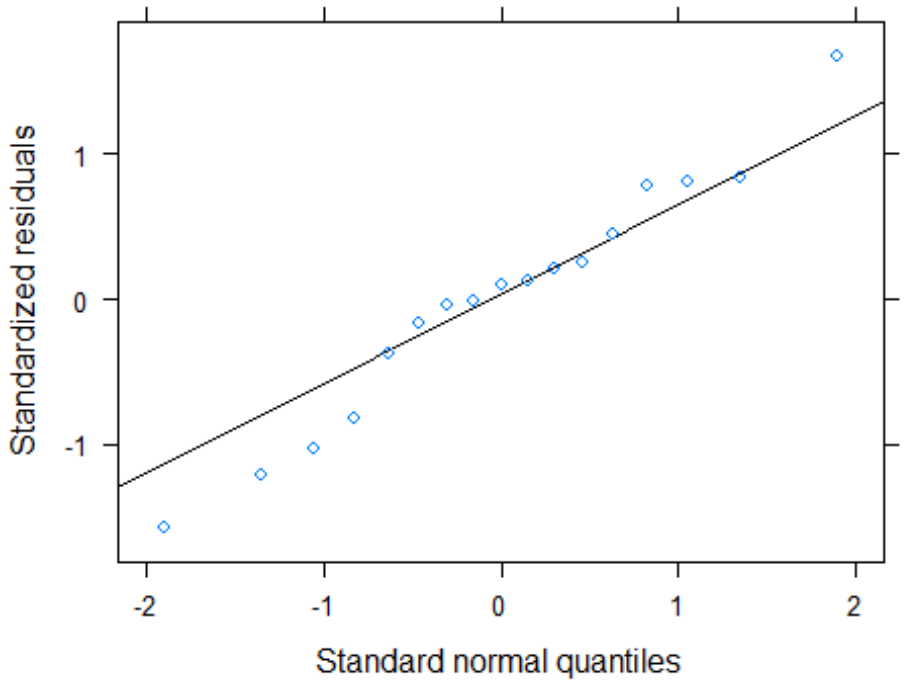
```
confint.merMod(STAGEAMODELP305,level=0.95,method="Wald")
```

```
##                    2.5 %       97.5 %
## .sig01                NA           NA
## .sigma                NA           NA
## (Intercept) 19.201330794 22.444821644
## VAP         -0.008476975  0.008110861
## SpermCount   0.001180993  0.005052077
## StatusS     -0.781942534 -0.160432486
```

```
plot(STAGEAMODELP305, results="hide", fig.show='hide')#Visual Check Variance
assumption
```




```
qqmath(STAGEAMODELP305)#Visual Check Normality assumption
```



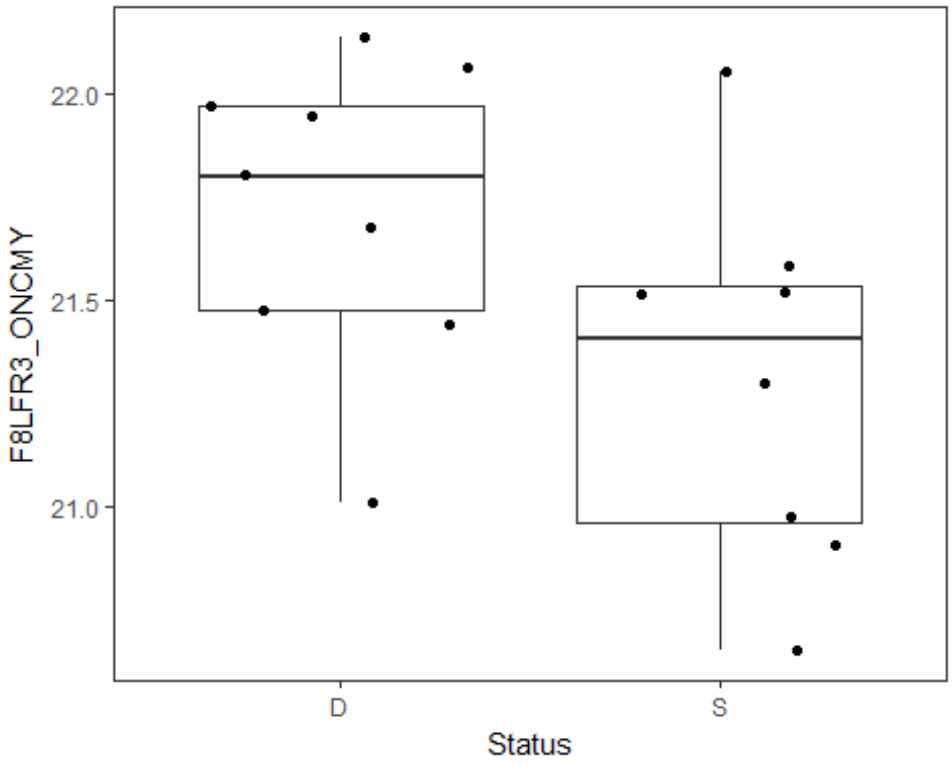

```
shapiro.test(resid(STAGEAMODELP305))#Test Check Normality assumption
```

```
##
##  Shapiro-Wilk normality test
##
## data:  resid(STAGEAMODELP305)
## W = 0.97294, p-value = 0.8677
```

```
rand(STAGEAMODELP305)#Test for significance of random predictor male ID
```

```
## Analysis of Random effects Table:
##      Chi.sq Chi.DF p.value
## Week   1.11      1     0.3
```

```
#Plot by status
ggplot(data=StageAonly, aes(x=Status, y=F8LFR3_ONCMY)) +
geom_boxplot()+ geom_jitter()+ theme_bw()+
theme(panel.grid.minor=element_blank(),panel.grid.major=element_blank())
```




```
proteins[[318]]
```

```
## [1] "Q64HX9_ONCMY"
```

```
STAGEAMODELP318<-lmer(Q64HX9_ONCMY ~ rescale(VAP) + rescale(SpermCount) + Sta
tus +
         + (1|Week),data=StageAonly)
summary(STAGEAMODELP318)
```

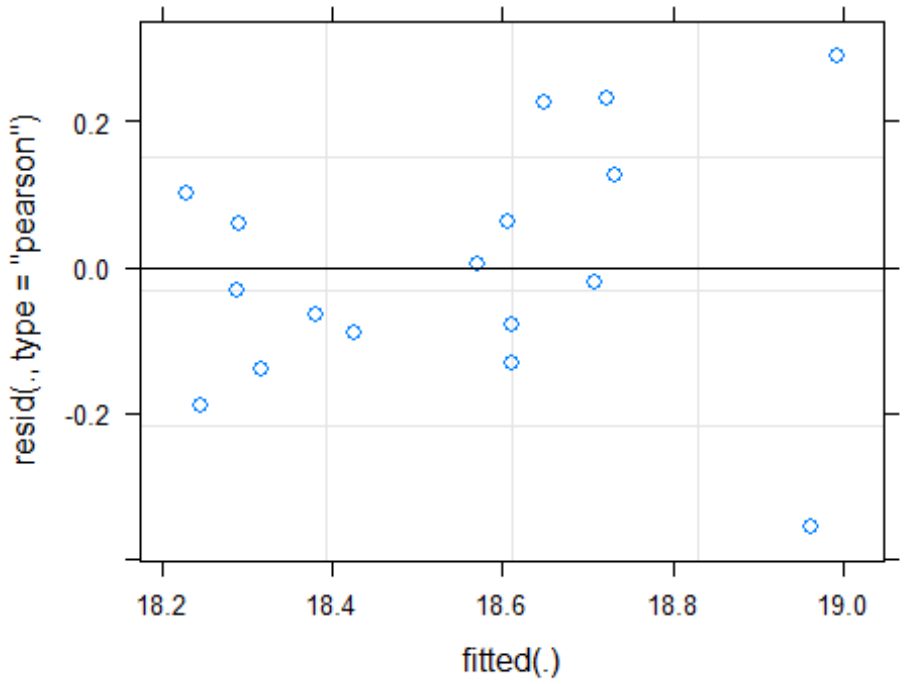
```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
```

```
## Formula: Q64HX9_ONCMY ~ VAP + SpermCount + Status + +(1 | Week)
##    Data: StageAonly
##
## REML criterion at convergence: 26.2
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.70340 -0.42901 -0.09665  0.48386  1.38999
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  Week     (Intercept) 0.04476  0.2116
##  Residual             0.04390  0.2095
## Number of obs: 17, groups:  Week, 5
##
## Fixed effects:
##               Estimate Std. Error         df t value Pr(>|t|)
## (Intercept)  1.868e+01  5.956e-01  1.141e+01  31.368 1.96e-12 ***
## VAP         -1.639e-03  3.049e-03  1.122e+01  -0.538   0.6013
## SpermCount  -7.287e-05  6.937e-04  1.030e+01  -0.105   0.9184
## StatusS      3.184e-01  1.114e-01  1.036e+01   2.858   0.0165 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn
## VAP         -0.919
## SpermCount -0.486  0.164
## StatusS     0.152 -0.165 -0.252
```

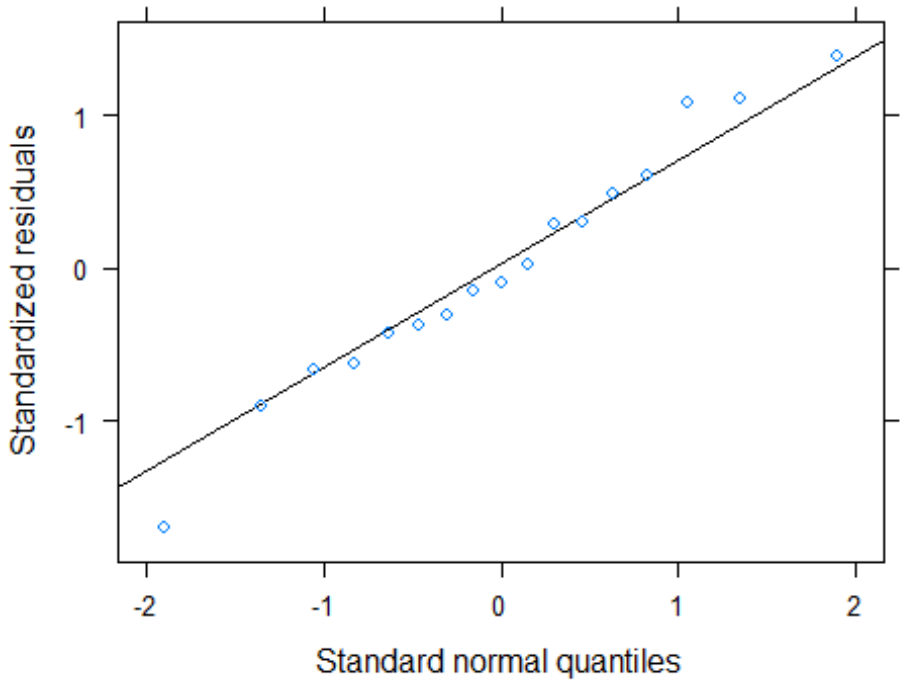
```
confint.merMod(STAGEAMODELP318,level=0.95,method="Wald")
```

```
##                    2.5 %       97.5 %
## .sig01                NA           NA
## .sigma                NA           NA
## (Intercept) 17.515257984 19.849974586
## VAP         -0.007615461  0.004336765
## SpermCount  -0.001432548  0.001286812
## StatusS      0.100084824  0.536808825
```

```
plot(STAGEAMODELP318, results="hide", fig.show='hide')#Visual Check Variance
assumption
```




```
qqmath(STAGEAMODELP318)#Visual Check Normality assumption
```



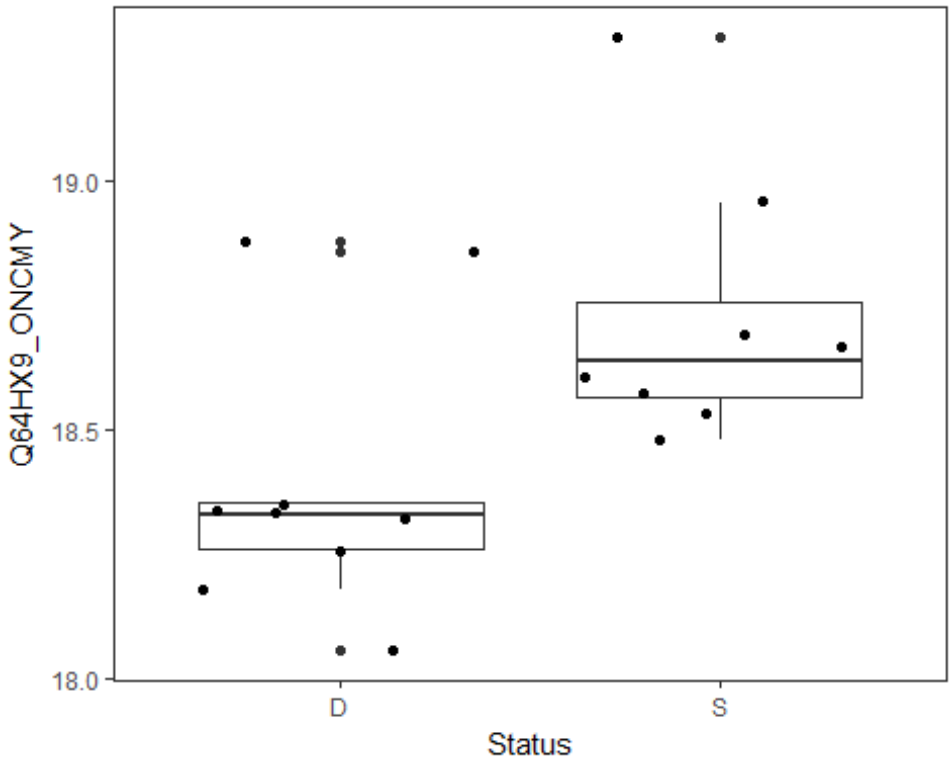

```
shapiro.test(resid(STAGEAMODELP318))#Test Check Normality assumption
```

```
##
##  Shapiro-Wilk normality test
##
## data:  resid(STAGEAMODELP318)
## W = 0.9792, p-value = 0.9495
```

```
rand(STAGEAMODELP318)#Test for significance of random predictor male ID
```

```
## Analysis of Random effects Table:
##      Chi.sq Chi.DF p.value
## Week   3.57      1    0.06 .
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
#Plot by status
ggplot(data=StageAonly, aes(x=Status, y=Q64HX9_ONCMY)) +
geom_boxplot()+ geom_jitter()+ theme_bw()+
theme(panel.grid.minor=element_blank(),panel.grid.major=element_blank())
```




```
proteins[[346]]
```

```
## [1] "W5S0H9_ONCMY"
```

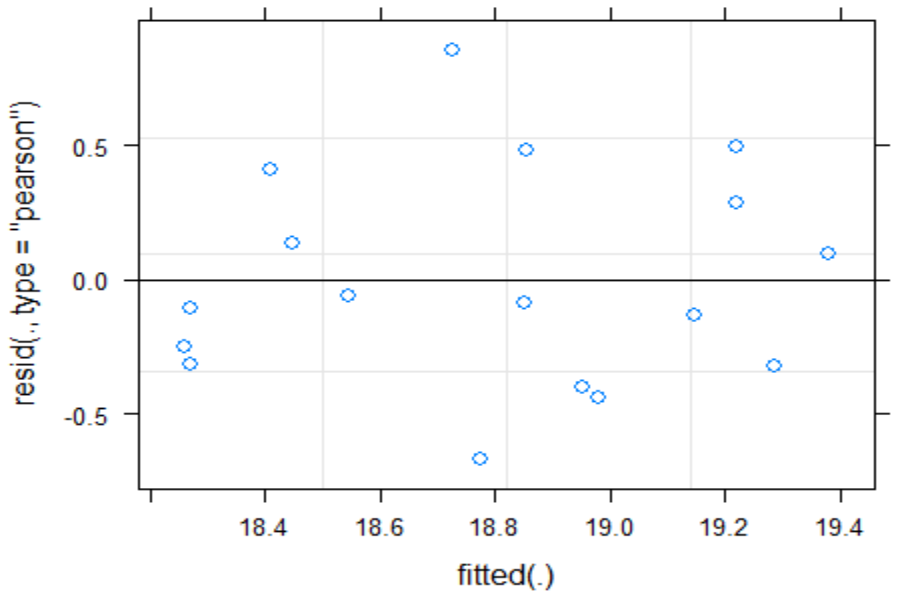
```
STAGEAMODELP346<-lmer(W5S0H9_ONCMY ~ rescale(VAP) + rescale(SpermCount) + Sta
tus +
         + (1|Week),data=StageAonly)
summary(STAGEAMODELP346)
```

```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: W5S0H9_ONCMY ~ VAP + SpermCount + Status + +(1 | Week)
##    Data: StageAonly
##
## REML criterion at convergence: 42.5
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.4481 -0.6860 -0.1790  0.6262  1.8545
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  Week     (Intercept) 0.0219   0.1480
##  Residual             0.2140   0.4626
## Number of obs: 17, groups:  Week, 5
##
## Fixed effects:
##              Estimate Std. Error        df t value Pr(>|t|)
## (Intercept) 18.671240   1.186139 12.996000  15.741 7.63e-10 ***
## VAP         -0.001212   0.006019 12.869000  -0.201   0.8436
## SpermCount   0.002143   0.001456 11.455000   1.472   0.1679
## StatusS     -0.673091   0.234315 11.043000  -2.873   0.0151 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn
## VAP         -0.922
## SpermCount -0.517  0.176
## StatusS     0.130 -0.171 -0.191
```

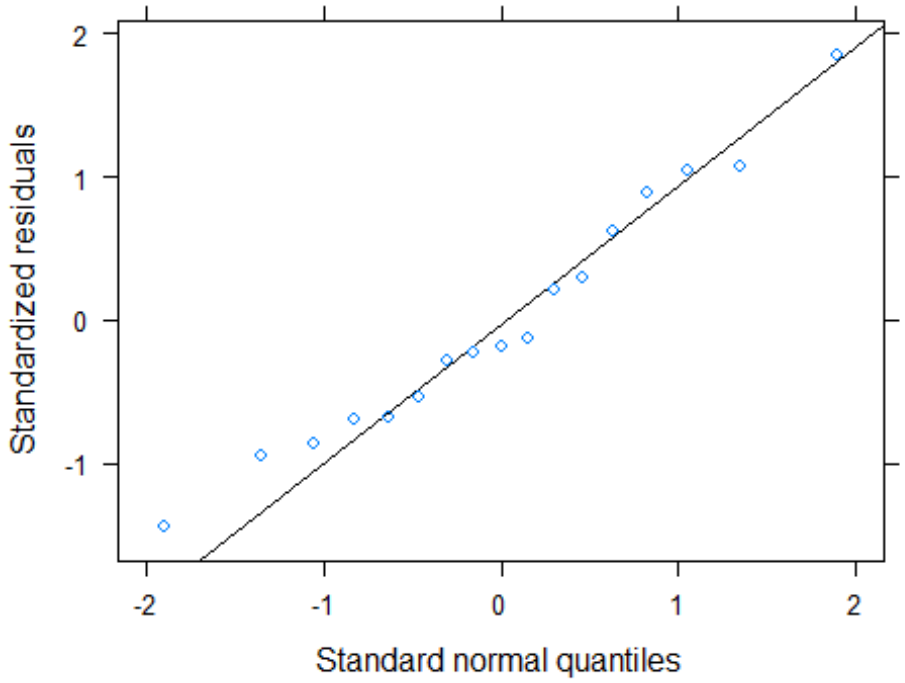
```
confint.merMod(STAGEAMODELP346,level=0.95,method="Wald")
```

```
##                     2.5 %       97.5 %
## .sig01                 NA           NA
## .sigma                 NA           NA
## (Intercept) 16.3464509519 20.996029756
## VAP         -0.0130087137  0.010584795
## SpermCount  -0.0007101128  0.004995682
## StatusS     -1.1323407353 -0.213841312
```

```
plot(STAGEAMODELP346, results="hide", fig.show='hide')#Visual Check Variance
assumption
```

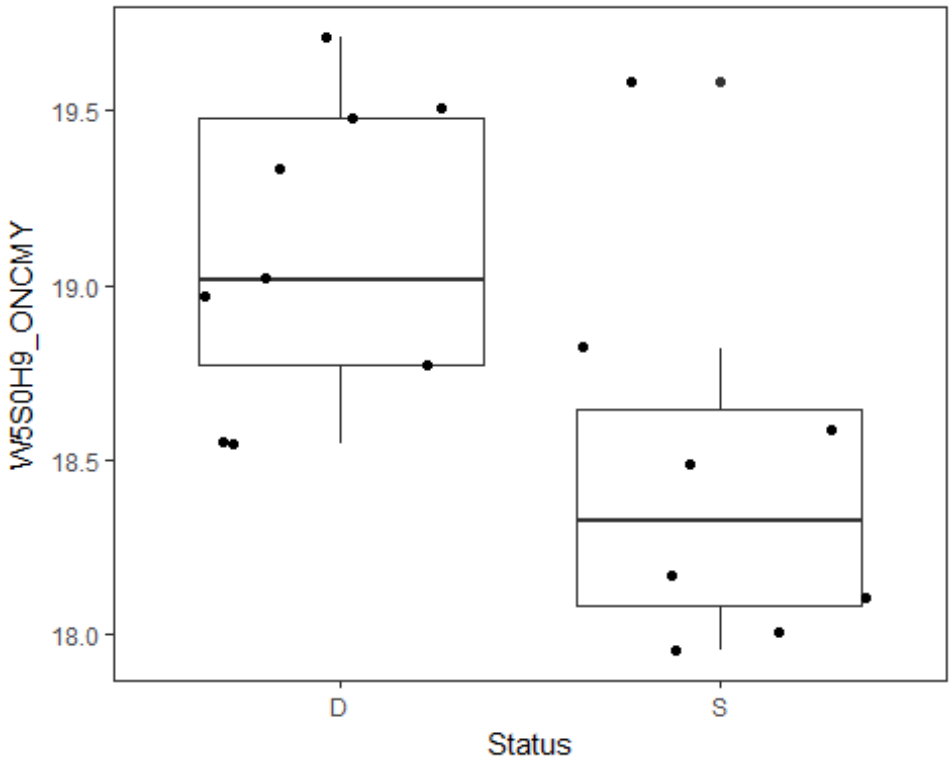



```
qqmath(STAGEAMODELP346)#Visual Check Normality assumption
```




```
shapiro.test(resid(STAGEAMODELP346))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
```

```
## data:  resid(STAGEAMODELP346)
## W = 0.96875, p-value = 0.7962

rand(STAGEAMODELP346)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##      Chi.sq Chi.DF p.value
## Week  0.079      1     0.8

#Plot by status
ggplot(data=StageAonly, aes(x=Status, y=W5S0H9_ONCMY)) +
geom_boxplot()+ geom_jitter()+ theme_bw()+
theme(panel.grid.minor=element_blank(),panel.grid.major=element_blank())
```



COMPARING DOMINANT AND SUBDOMINANT MALES STAGE 2: models with status as significant predictor are shown below. VAP and sperm number were included as fixed effects in these models

```
proteins[[12]]
```

```
## [1] "H2B_ONCMY"
```

```
STAGEBMODELP12<-lmer(H2B_ONCMY ~ rescale(VAP) + rescale(SpermCount) + Status
+
                     + (1|Week),data=StageBonly)
summary(STAGEBMODELP12)
```

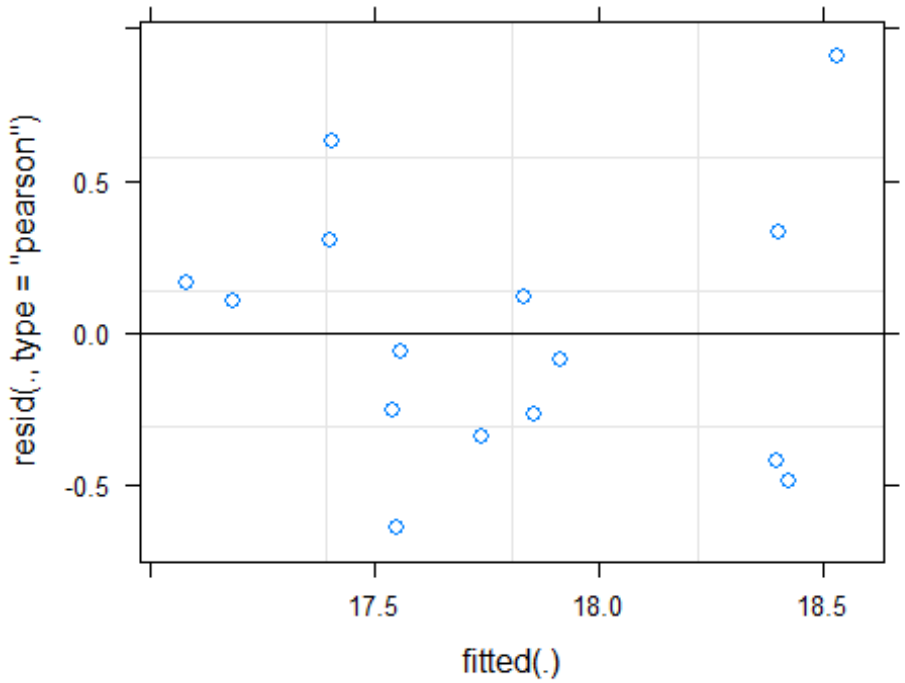
```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: H2B_ONCMY ~ VAP + SpermCount + Status + +(1 | Week)
##    Data: StageBonly
##
## REML criterion at convergence: 38.8
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.3279 -0.6266 -0.1326  0.4876  1.8840
##
## Random effects:
##  Groups   Name        Variance  Std.Dev.
##  Week     (Intercept) 2.468e-17 4.968e-09
##  Residual             2.345e-01 4.842e-01
## Number of obs: 15, groups:  Week, 5
##
## Fixed effects:
##              Estimate Std. Error        df t value Pr(>|t|)
## (Intercept) 16.428488   1.911484 11.000000   8.595 3.28e-06 ***
## VAP          0.010706   0.009630 11.000000   1.112   0.2900
## SpermCount  -0.002333   0.001525 11.000000  -1.530   0.1543
## StatusS      0.746872   0.271119 11.000000   2.755   0.0187 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn
## VAP         -0.969
## SpermCount -0.610  0.411
## StatusS     0.237 -0.239 -0.316
```

```
confint.merMod(STAGEBMODELP12,level=0.95,method="Wald")
```

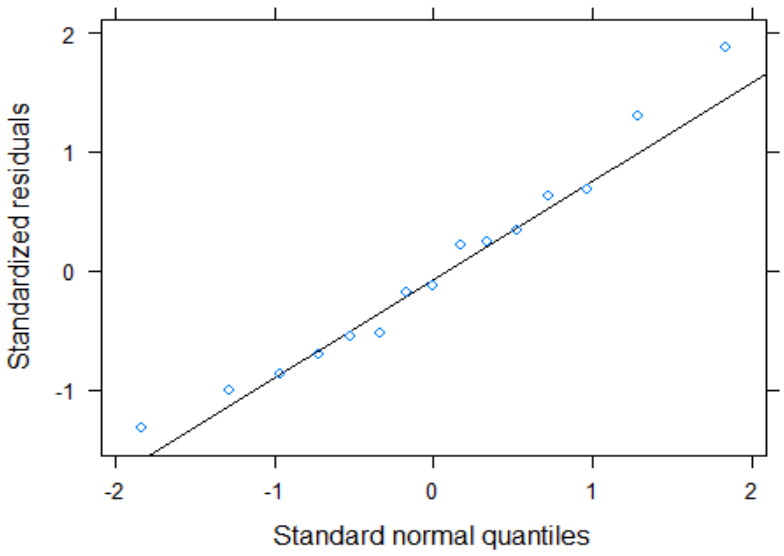
```
##                    2.5 %       97.5 %
## .sig01                NA           NA
## .sigma                NA           NA
## (Intercept) 12.682048375 2.017493e+01
```

```
## VAP          -0.008168350 2.958062e-02
## SpermCount   -0.005322953 6.564615e-04
## StatusS       0.215488712 1.278255e+00
```

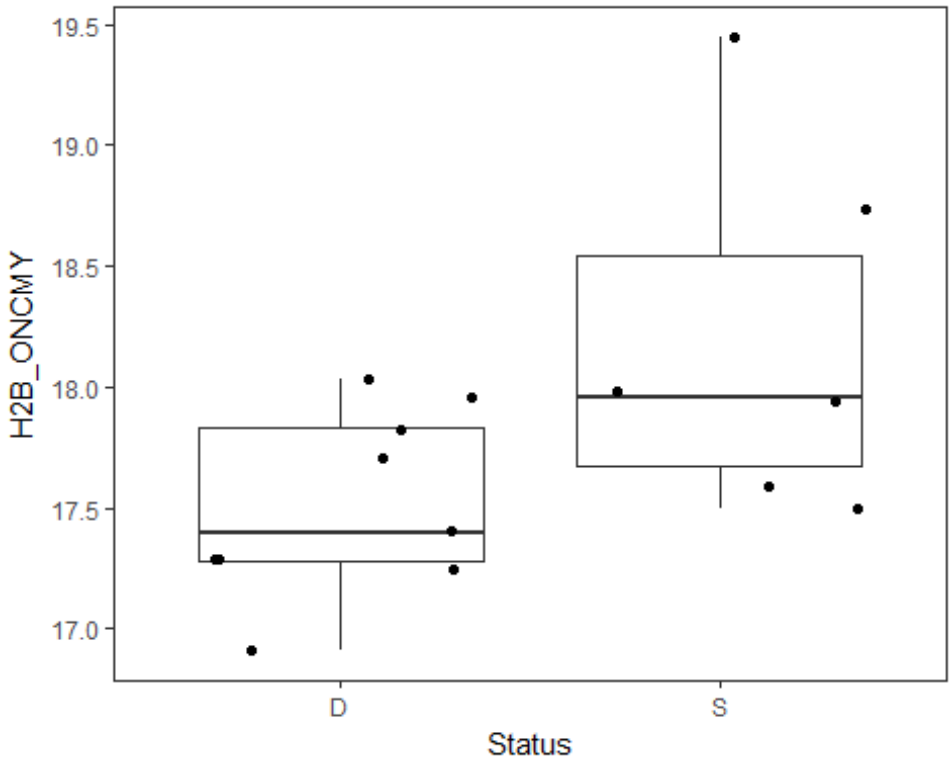
```
plot(STAGEBMODELP12, results="hide", fig.show='hide')#Visual Check Variance a
ssumption
```




```
qqmath(STAGEBMODELP12)#Visual Check Normality assumption
```

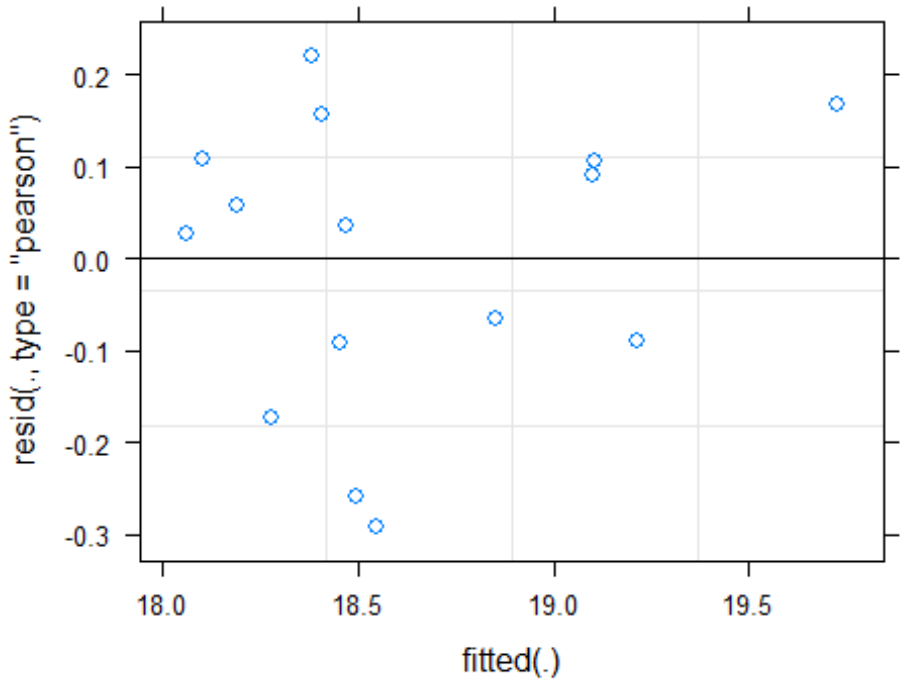


```
shapiro.test(resid(STAGEBMODELP12))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
## data:  resid(STAGEBMODELP12)
## W = 0.96802, p-value = 0.8277

rand(STAGEBMODELP12)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##         Chi.sq Chi.DF p.value
## Week -7.11e-14      1       1

summarySE(data=StageBonly, measurevar = "H2B_ONCMY", groupvars = "Status", co
nf.interval = .095)

##   Status N H2B_ONCMY        sd        se         ci
## 1      D 9  17.51841 0.3800238 0.1266746 0.01560427
## 2      S 6  18.19615 0.7519260 0.3069725 0.03853229

#Plot by status
ggplot(data=StageBonly, aes(x=Status, y=H2B_ONCMY)) +
  geom_boxplot()+ geom_jitter()+ theme_bw()+
  theme(panel.grid.minor=element_blank(),panel.grid.major=element_blank())
```

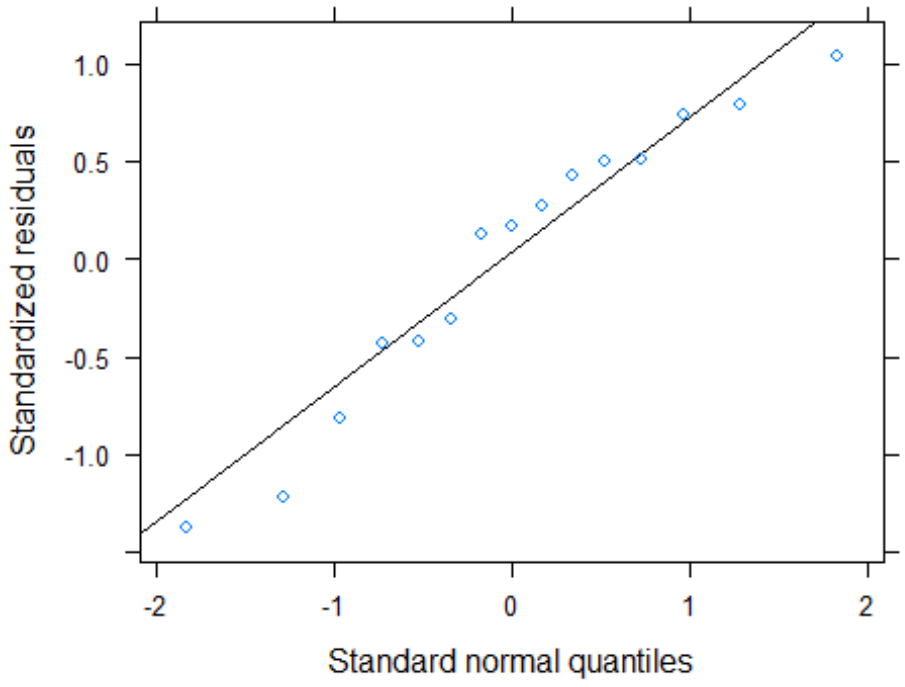



```
proteins[[46]]
```

```
## [1] "B5DGY4_SALSA"

STAGEBMODELP46<-lmer(B5DGY4_SALSA ~ rescale(VAP) + rescale(SpermCount) + Stat
us +
                     + (1|Week),data=StageBonly)
summary(STAGEBMODELP46)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: B5DGY4_SALSA ~ VAP + SpermCount + Status + +(1 | Week)
##    Data: StageBonly
##
## REML criterion at convergence: 28.9
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.3778 -0.4281  0.1692  0.5020  1.0415
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  Week     (Intercept) 0.16939  0.4116
##  Residual             0.04516  0.2125
## Number of obs: 15, groups:  Week, 5
##
## Fixed effects:
##               Estimate Std. Error         df t value Pr(>|t|)
## (Intercept) 22.6605716  1.5332830 10.4180000  14.779 2.52e-08 ***
## VAP         -0.0153578  0.0077070 10.2750000  -1.993  0.07352 .
## SpermCount  -0.0045506  0.0009878  9.4550000  -4.607  0.00112 **
## StatusS      0.4135034  0.1285419  8.0350000   3.217  0.01222 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn
## VAP         -0.979
## SpermCount -0.719  0.604
## StatusS     0.378 -0.364 -0.457

confint.merMod(STAGEBMODELP46,level=0.95,method="Wald")

##                    2.5 %        97.5 %
## .sig01                NA            NA
## .sigma                NA            NA
## (Intercept) 19.655392032 25.6657511372
## VAP         -0.030463214 -0.0002523683
## SpermCount  -0.006486767 -0.0026144869
## StatusS      0.161565989  0.6654408743

plot(STAGEBMODELP46, results="hide", fig.show='hide')#Visual Check Variance a
ssumption
```




```
qqmath(STAGEBMODELP46)#Visual Check Normality assumption
```




```
shapiro.test(resid(STAGEBMODELP46))#Test Check Normality assumption
```

```
##
##  Shapiro-Wilk normality test
##
## data:  resid(STAGEBMODELP46)
## W = 0.94116, p-value = 0.3971
```

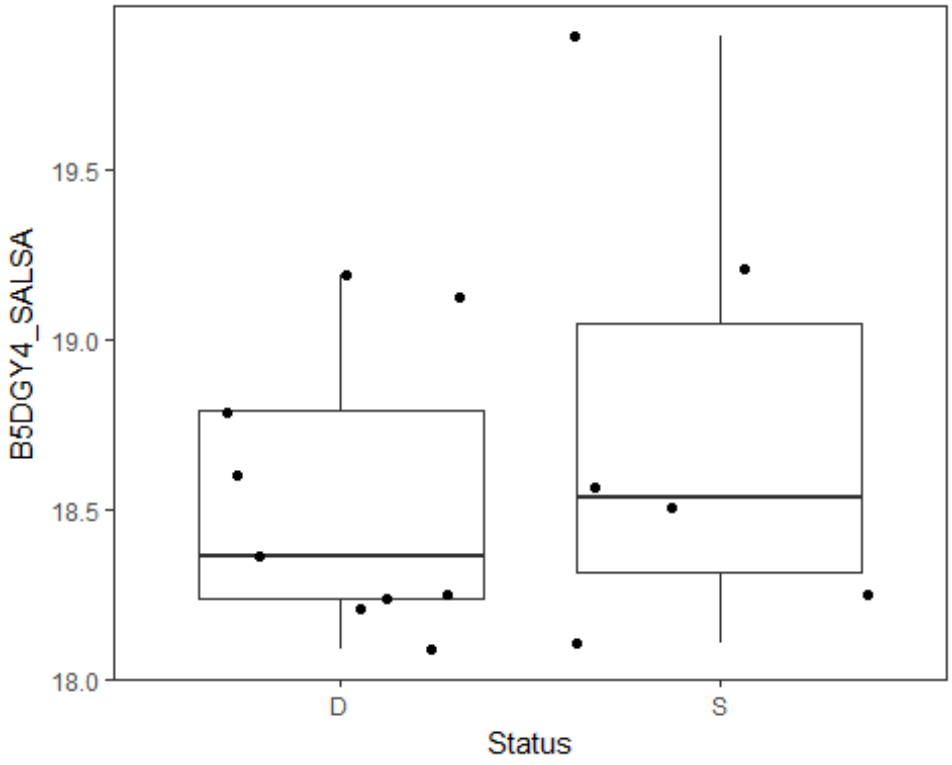
```
rand(STAGEBMODELP46)#Test for significance of random predictor male ID
```

```
## Analysis of Random effects Table:
##      Chi.sq Chi.DF p.value
## Week   8.44      1   0.004 **
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
summarySE(data=StageBonly, measurevar = "B5DGY4_SALSA", groupvars = "Status",
conf.interval = .095)
```

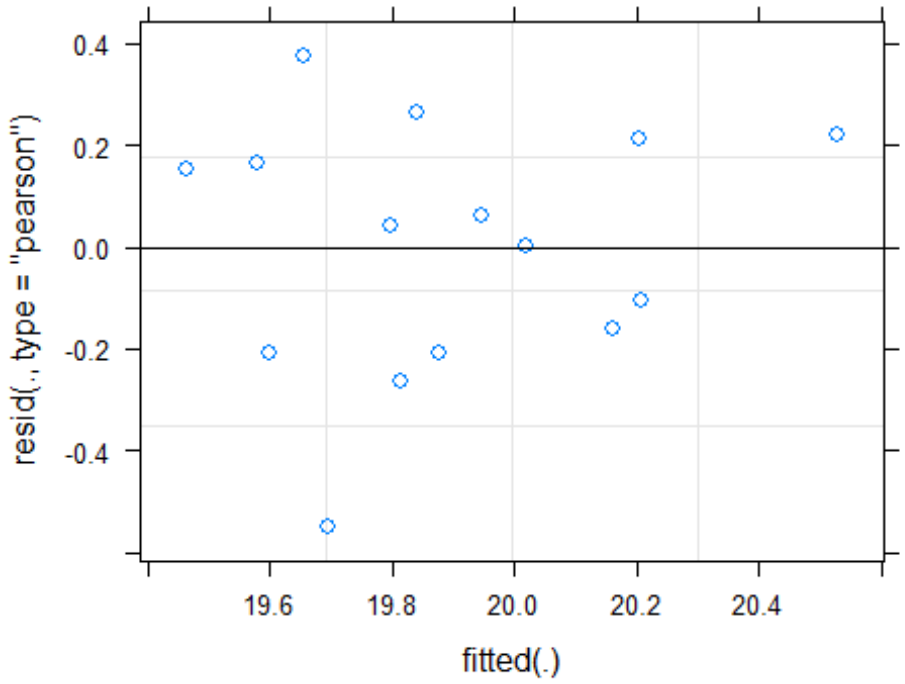
```
##   Status N B5DGY4_SALSA        sd        se         ci
## 1      D 9     18.53874 0.4118231 0.1372744 0.01691000
## 2      S 6     18.75476 0.6757422 0.2758706 0.03462827
```

```
#Plot by status
ggplot(data=StageBonly, aes(x=Status, y=B5DGY4_SALSA)) +
  geom_boxplot()+ geom_jitter()+ theme_bw()+
  theme(panel.grid.minor=element_blank(),panel.grid.major=element_blank())
```

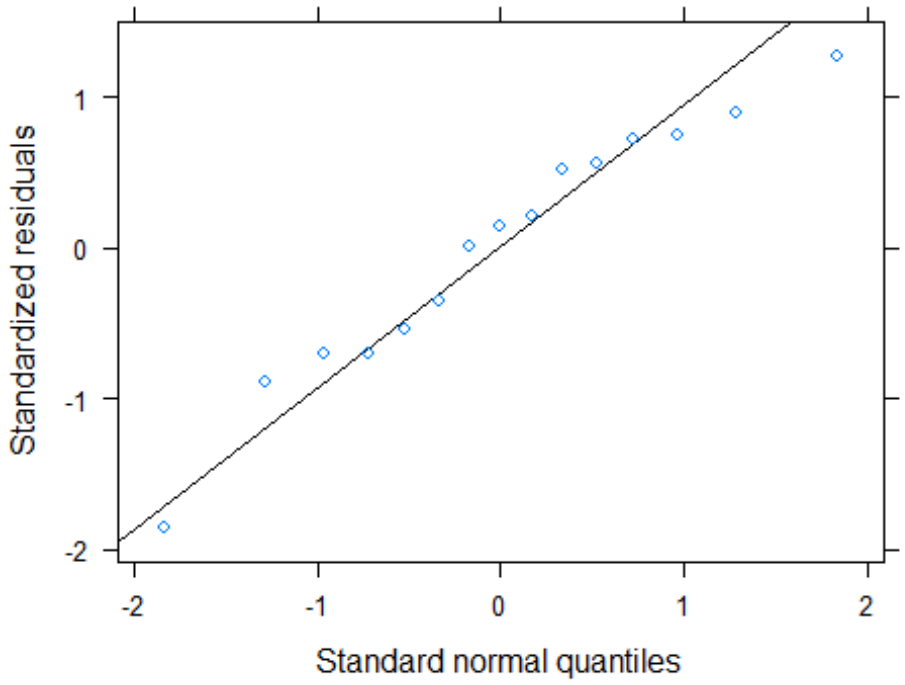



```
proteins[[74]]
```

```
## [1] "B5X202_SALSA"

STAGEBMODELP74<-lmer(B5X202_SALSA ~ rescale(VAP) + rescale(SpermCount) + Stat
us +
                     + (1|Week),data=StageBonly)
summary(STAGEBMODELP74)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: B5X202_SALSA ~ VAP + SpermCount + Status + +(1 | Week)
##    Data: StageBonly
##
## REML criterion at convergence: 29.4
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.8640 -0.6254  0.1446  0.6387  1.2747
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  Week     (Intercept) 0.01929  0.1389
##  Residual             0.08752  0.2958
## Number of obs: 15, groups:  Week, 5
##
## Fixed effects:
##              Estimate Std. Error        df t value Pr(>|t|)
## (Intercept) 22.371557   1.399842  6.576000  15.981 1.66e-06 ***
## VAP         -0.011593   0.007075  6.486000  -1.639   0.1487
## SpermCount  -0.002010   0.001048  9.937000  -1.918   0.0843 .
## StatusS      0.517699   0.168488  9.298000   3.073   0.0128 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn
## VAP         -0.974
## SpermCount -0.635  0.459
## StatusS     0.264 -0.258 -0.349

confint.merMod(STAGEBMODELP74,level=0.95,method="Wald")

##                    2.5 %       97.5 %
## .sig01                NA           NA
## .sigma                NA           NA
## (Intercept) 19.627917035 2.511520e+01
## VAP         -0.025459270 2.272789e-03
## SpermCount  -0.004065012 4.422970e-05
## StatusS      0.187469091 8.479282e-01

plot(STAGEBMODELP74, results="hide", fig.show='hide')#Visual Check Variance a
ssumption
```




```
qqmath(STAGEBMODELP74)#Visual Check Normality assumption
```




```
shapiro.test(resid(STAGEBMODELP74))#Test Check Normality assumption
```

```
##
##  Shapiro-Wilk normality test
##
## data:  resid(STAGEBMODELP74)
## W = 0.95934, p-value = 0.6809
```

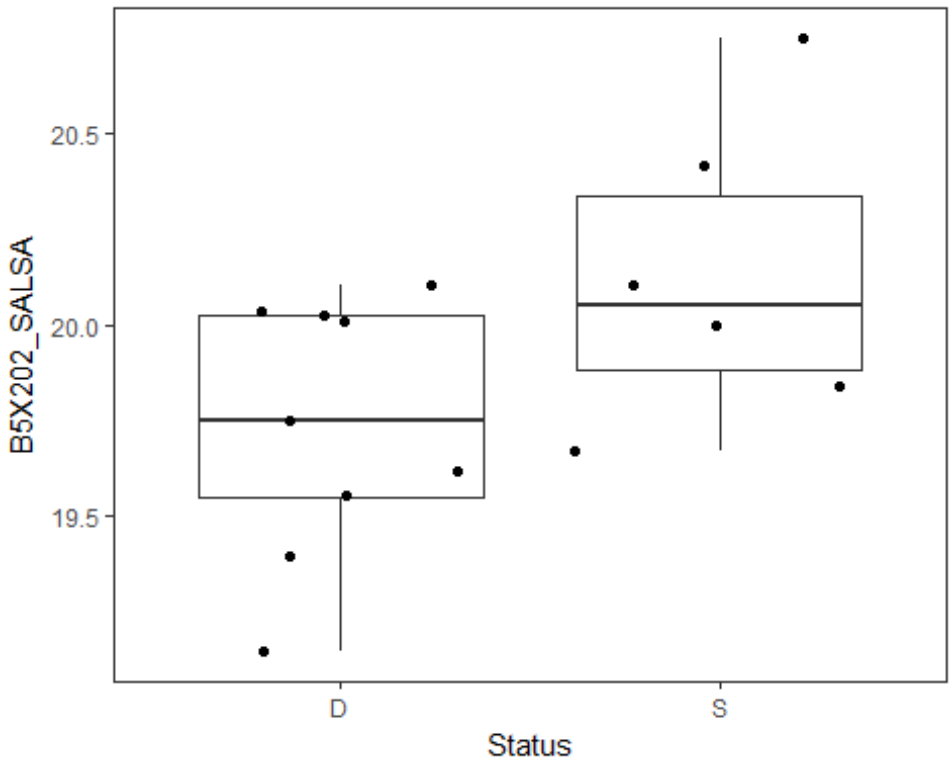
```
rand(STAGEBMODELP74)#Test for significance of random predictor male ID
```

```
## Analysis of Random effects Table:
##      Chi.sq Chi.DF p.value
## Week  0.326      1     0.6
```

```
summarySE(data=StageBonly, measurevar = "B5X202_SALSA", groupvars = "Status",
conf.interval = .095)
```

```
##   Status N B5X202_SALSA        sd        se         ci
## 1      D 9     19.73499 0.3354651 0.1118217 0.01377464
## 2      S 6     20.12914 0.3952995 0.1613804 0.02025704
```

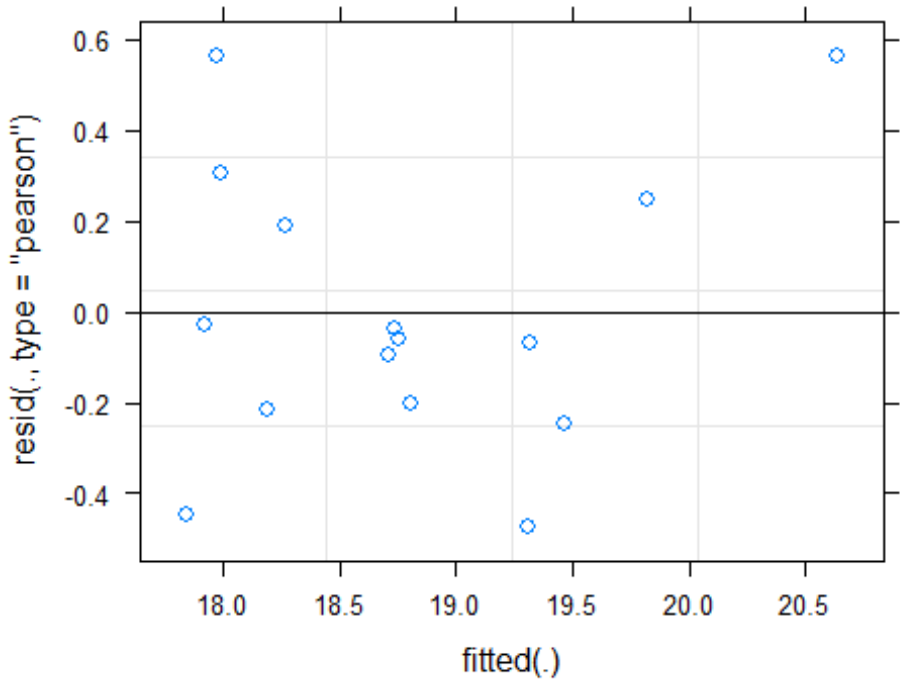
```
#Plot by status
ggplot(data=StageBonly, aes(x=Status, y=B5X202_SALSA)) +
  geom_boxplot()+ geom_jitter()+ theme_bw()+
  theme(panel.grid.minor=element_blank(),panel.grid.major=element_blank())
```



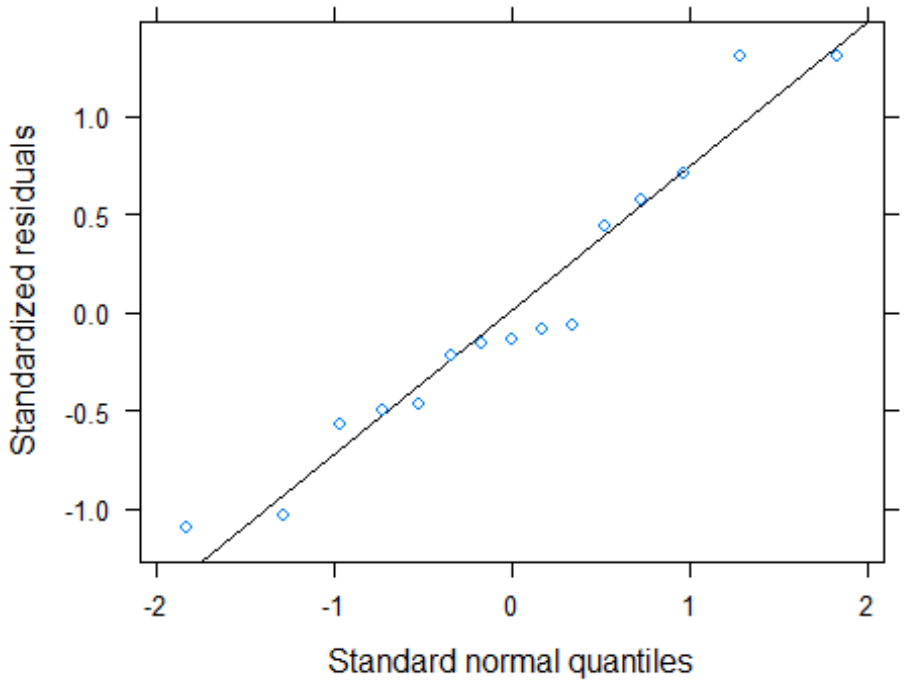

```
proteins[[99]]
```

```
## [1] "B5X4I3_SALSA"
```

```
STAGEBMODELP99<-lmer(B5X4I3_SALSA ~ rescale(VAP) + rescale(SpermCount) + Stat
us +
                     + (1|Week),data=StageBonly)
summary(STAGEBMODELP99)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: B5X4I3_SALSA ~ VAP + SpermCount + Status + +(1 | Week)
##    Data: StageBonly
##
## REML criterion at convergence: 44.3
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.1010 -0.4810 -0.1351  0.5049  1.3089
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  Week     (Intercept) 0.6675   0.817
##  Residual             0.1867   0.432
## Number of obs: 15, groups:  Week, 5
##
## Fixed effects:
##              Estimate Std. Error        df t value Pr(>|t|)
## (Intercept) 1.405e+01  3.102e+00 1.027e+01   4.530  0.00102 **
## VAP         2.412e-02  1.560e-02 1.009e+01   1.547  0.15270
## SpermCount  9.342e-04  2.002e-03 8.984e+00   0.467  0.65190
## StatusS     8.611e-01  2.611e-01 7.194e+00   3.298  0.01266 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn
## VAP         -0.979
## SpermCount -0.718  0.602
## StatusS     0.376 -0.362 -0.456

confint.merMod(STAGEBMODELP99,level=0.95,method="Wald")

##                    2.5 %       97.5 %
## .sig01                NA           NA
## .sigma                NA           NA
## (Intercept)  7.970959366 20.130921124
## VAP         -0.006445991  0.054694496
## SpermCount  -0.002990093  0.004858517
## StatusS      0.349310831  1.372875264

plot(STAGEBMODELP99, results="hide", fig.show='hide')#Visual Check Variance a
ssumption
```




```
qqmath(STAGEBMODELP99)#Visual Check Normality assumption
```




```
shapiro.test(resid(STAGEBMODELP99))#Test Check Normality assumption
```

```
##
##  Shapiro-Wilk normality test
##
## data:  resid(STAGEBMODELP99)
## W = 0.93859, p-value = 0.365
```

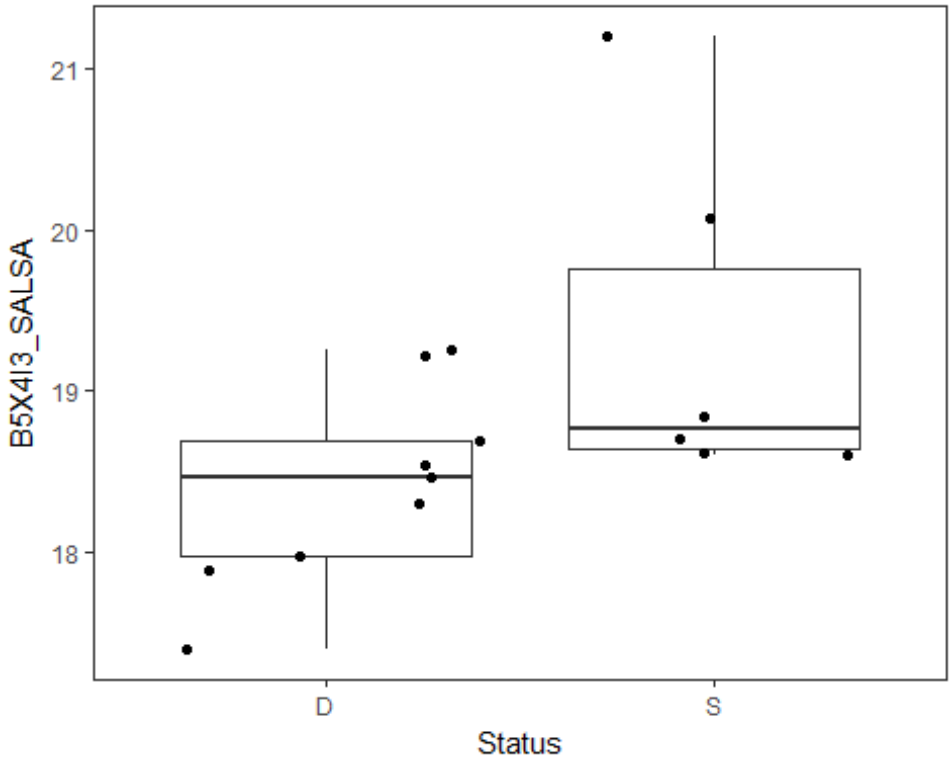
```
rand(STAGEBMODELP99)#Test for significance of random predictor male ID
```

```
## Analysis of Random effects Table:
##      Chi.sq Chi.DF p.value
## Week   3.95      1    0.05 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
summarySE(data=StageBonly, measurevar = "B5X4I3_SALSA", groupvars = "Status",
conf.interval = .095)
```

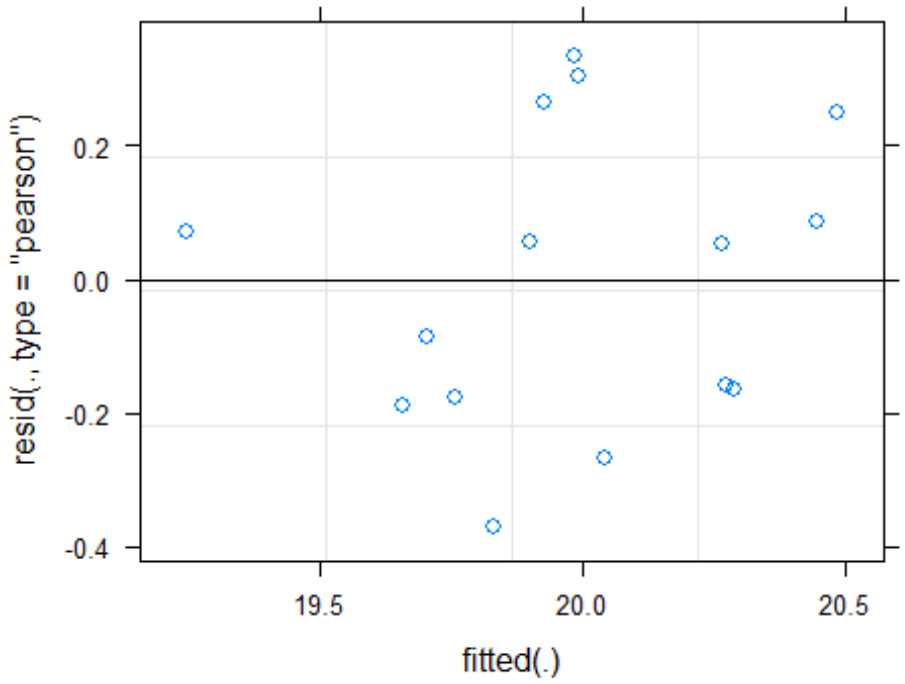
```
##   Status N B5X4I3_SALSA        sd        se         ci
## 1      D 9     18.41222 0.6091064 0.2030355 0.02501071
## 2      S 6     19.33874 1.0710059 0.4372363 0.05488347
```

```
#Plot by status
ggplot(data=StageBonly, aes(x=Status, y=B5X4I3_SALSA)) +
  geom_boxplot()+ geom_jitter()+ theme_bw()+
  theme(panel.grid.minor=element_blank(),panel.grid.major=element_blank())
```

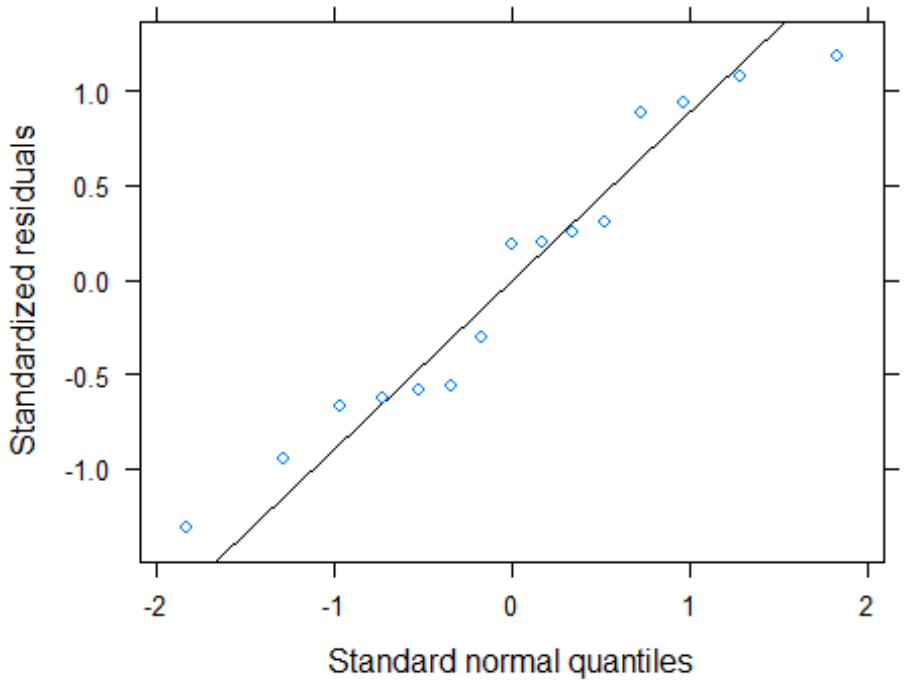



```
proteins[[339]]
```

```
## [1] "Q9DFG0_ONCMY"

STAGEBMODELP339<-lmer(Q9DFG0_ONCMY ~ rescale(VAP) + rescale(SpermCount) + Sta
tus +
                      + (1|Week),data=StageBonly)
summary(STAGEBMODELP339)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: Q9DFG0_ONCMY ~ VAP + SpermCount + Status + +(1 | Week)
##    Data: StageBonly
##
## REML criterion at convergence: 30.6
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.3166 -0.6069  0.1845  0.5961  1.1887
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  Week     (Intercept) 0.06387  0.2527
##  Residual             0.07950  0.2820
## Number of obs: 15, groups:  Week, 5
##
## Fixed effects:
##              Estimate Std. Error        df t value Pr(>|t|)
## (Intercept) 16.230774   1.634963  9.324000   9.927 2.91e-06 ***
## VAP          0.017606   0.008265  9.384000   2.130   0.0608 .
## SpermCount   0.002631   0.001139 10.993000   2.310   0.0413 *
## StatusS     -0.450238   0.164642  8.533000  -2.735   0.0242 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn
## VAP         -0.978
## SpermCount -0.672  0.525
## StatusS     0.312 -0.300 -0.397

confint.merMod(STAGEBMODELP339,level=0.95,method="Wald")

##                    2.5 %      97.5 %
## .sig01                NA          NA
## .sigma                NA          NA
## (Intercept) 13.026304799 19.43524311
## VAP          0.001406785  0.03380452
## SpermCount   0.000398640  0.00486372
## StatusS     -0.772929964 -0.12754509

plot(STAGEBMODELP339, results="hide", fig.show='hide')#Visual Check Variance
assumption
```




```
qqmath(STAGEBMODELP339)#Visual Check Normality assumption
```




```
shapiro.test(resid(STAGEBMODELP339))#Test Check Normality assumption
```

```
##
##  Shapiro-Wilk normality test
##
## data:  resid(STAGEBMODELP339)
## W = 0.94097, p-value = 0.3947
```

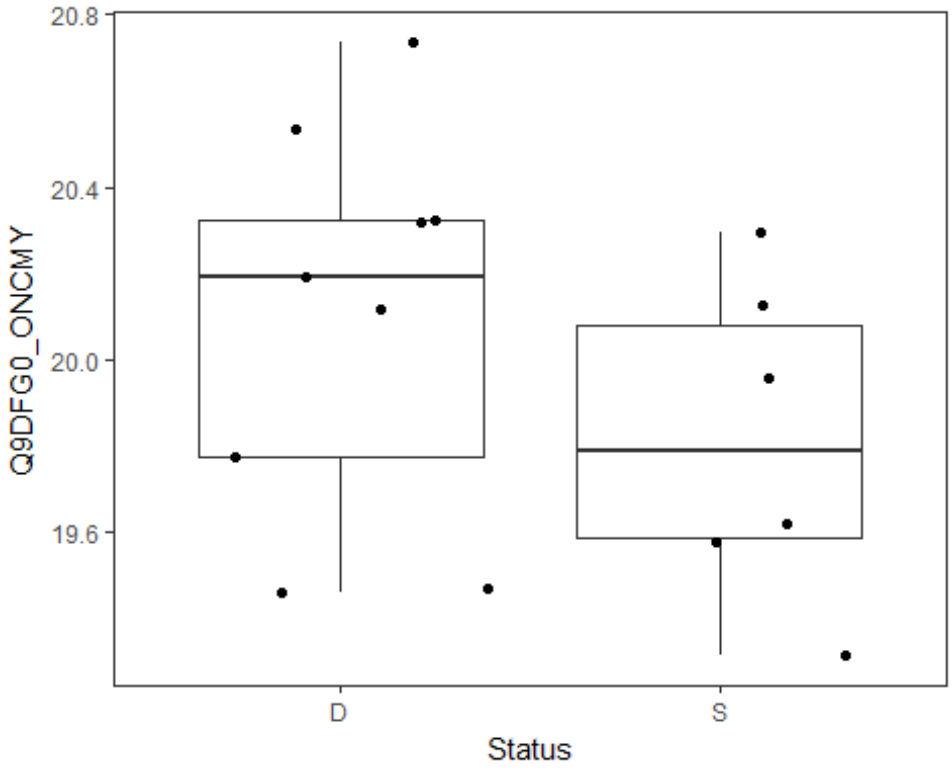
```
rand(STAGEBMODELP339)#Test for significance of random predictor male ID
```

```
## Analysis of Random effects Table:
##      Chi.sq Chi.DF p.value
## Week   1.48      1     0.2
```

```
summarySE(data=StageBonly, measurevar = "Q9DFG0_ONCMY", groupvars = "Status",
conf.interval = .095)
```

```
##   Status N Q9DFG0_ONCMY        sd        se         ci
## 1      D 9     20.10205 0.4497939 0.1499313 0.01846913
## 2      S 6     19.81365 0.3715105 0.1516685 0.01903798
```

```
#Plot by status
ggplot(data=StageBonly, aes(x=Status, y=Q9DFG0_ONCMY)) +
  geom_boxplot()+ geom_jitter()+ theme_bw()+
  theme(panel.grid.minor=element_blank(),panel.grid.major=element_blank())
```



COMPARING DOMINANT AND SUBDOMINANT MALES ACROSS BOTH STAGES: models with status as significant predictor are shown below. VAP and sperm number were included as fixed effects in these models, the experimental stage that the ejaculate was collected in was also included as a cofactor.

```
proteins[[7]]
```

```
## [1] "H1_ONCMY"
```

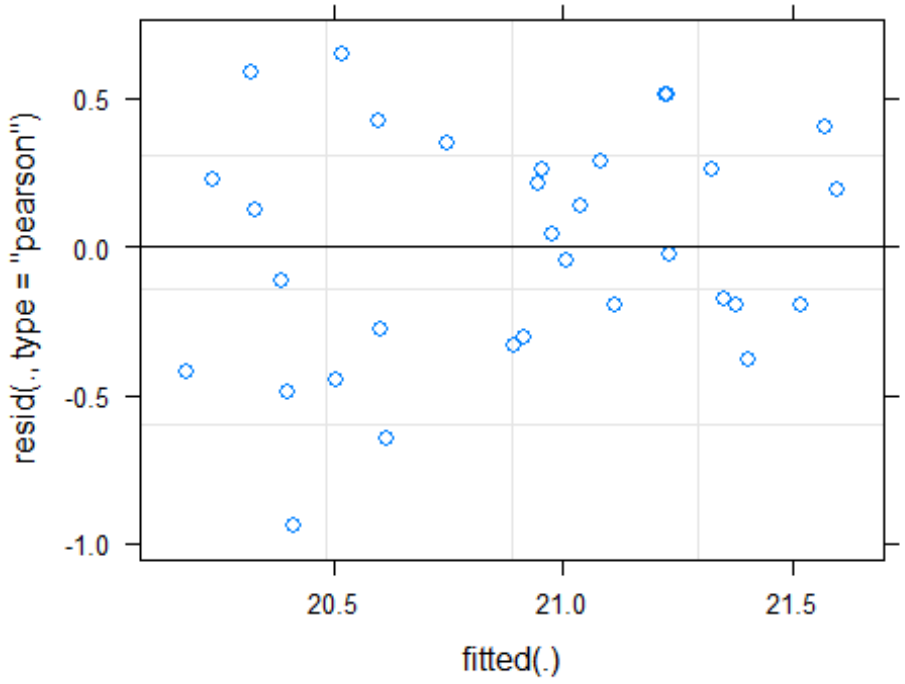
```
modelP7<-lmer(H1_ONCMY ~ rescale(VAP) + rescale(SpermCount) + Status + Stage
+
                  (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats
)
summary(modelP7)
```

```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: H1_ONCMY ~ rescale(VAP) + rescale(SpermCount) + Status + Stage +
##     (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 48.1
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -2.07435 -0.62178  0.01747  0.59103  1.43641
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.04934  0.2221
##  Week     (Intercept) 0.00000  0.0000
##  Residual             0.20680  0.4548
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error      df t value Pr(>|t|)
## (Intercept)          21.1446     0.3044 25.6080  69.469  < 2e-16 ***
## rescale(VAP)         -0.1627     0.3822 25.4030  -0.426  0.67400
## rescale(SpermCount)  -0.6014     0.3801 24.8470  -1.582  0.12626
## StatusS               0.6375     0.1878 26.9990   3.395  0.00214 **
## StageB               -0.4231     0.1660 13.7510  -2.549  0.02340 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.793
## rscl(SprmC) -0.580  0.297
## StatusS     -0.063 -0.171 -0.240
## StageB      -0.129 -0.095 -0.191  0.137
```

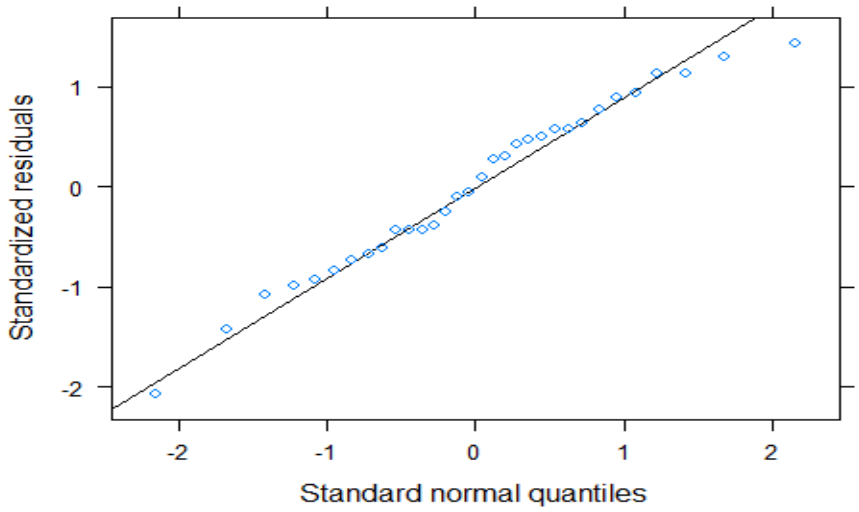
```
confint.merMod(modelP7,level=0.95,method="Wald")
```

```
##                            2.5 %      97.5 %
## .sig01                        NA          NA
## .sig02                        NA          NA
## .sigma                        NA          NA
## (Intercept)          20.5480482 21.74117396
## rescale(VAP)         -0.9117648  0.58645103
## rescale(SpermCount) -1.3464408  0.14361024
## StatusS               0.2694944  1.00559469
## StageB               -0.7483291 -0.09780101
```

```
plot(modelP7, results="hide", fig.show='hide')#Visual Check Variance assumpti
on
```




```
qqmath(modelP7)#Visual Check Normality assumption
```



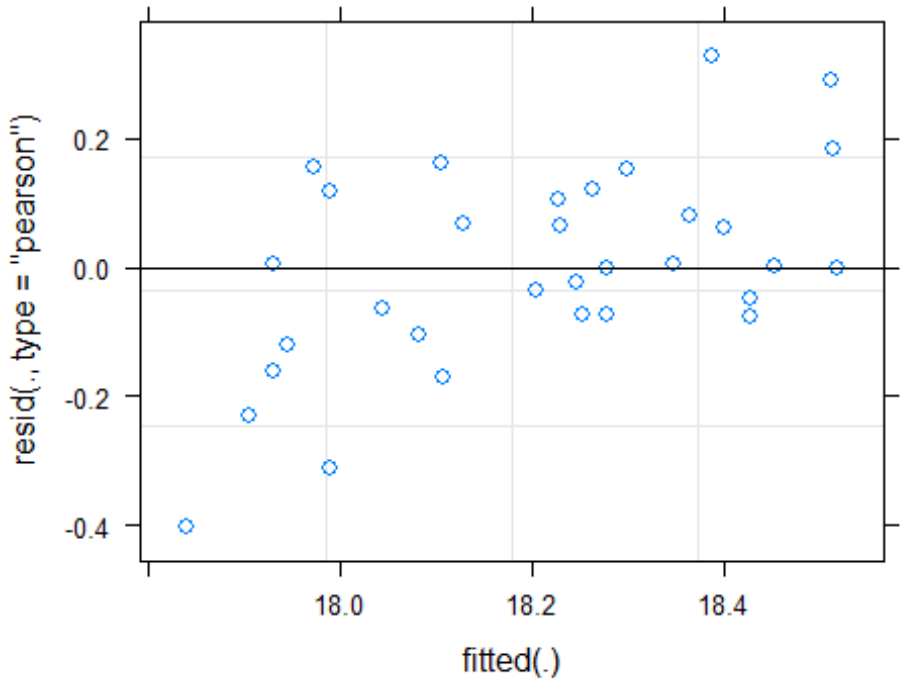

```
shapiro.test(resid(modelP7))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP7)
## W = 0.97608, p-value = 0.6804

rand(modelP7)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##          Chi.sq Chi.DF p.value
## MaleID 4.64e-01      1     0.5
## Week   2.84e-14      1     1.0

proteins[[40]]

## [1] "B5DGM5_SALSA"

modelP40<-lmer(B5DGM5_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status + S
tage +
                (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats)
summary(modelP40)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: B5DGM5_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
##     Stage + (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 23
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
```

```
## -1.83724 -0.33791 -0.00186  0.49059  1.49585
##
## Random effects:
##  Groups   Name        Variance  Std.Dev.
##  MaleID   (Intercept) 7.209e-02 2.685e-01
##  Week     (Intercept) 8.472e-18 2.911e-09
##  Residual             4.837e-02 2.199e-01
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error       df t value Pr(>|t|)
## (Intercept)         18.27527    0.19622 26.97300  93.136   <2e-16 ***
## rescale(VAP)        -0.17697    0.24224 25.45700  -0.731    0.472
## rescale(SpermCount) -0.08902    0.21307 15.31800  -0.418    0.682
## StatusS              0.24454    0.11042 17.85300   2.215    0.040 *
## StageB              -0.07931    0.08163  8.68900  -0.972    0.358
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.812
## rscl(SprmC) -0.582  0.374
## StatusS     -0.097 -0.116 -0.228
## StageB      -0.086 -0.099 -0.184  0.181
```

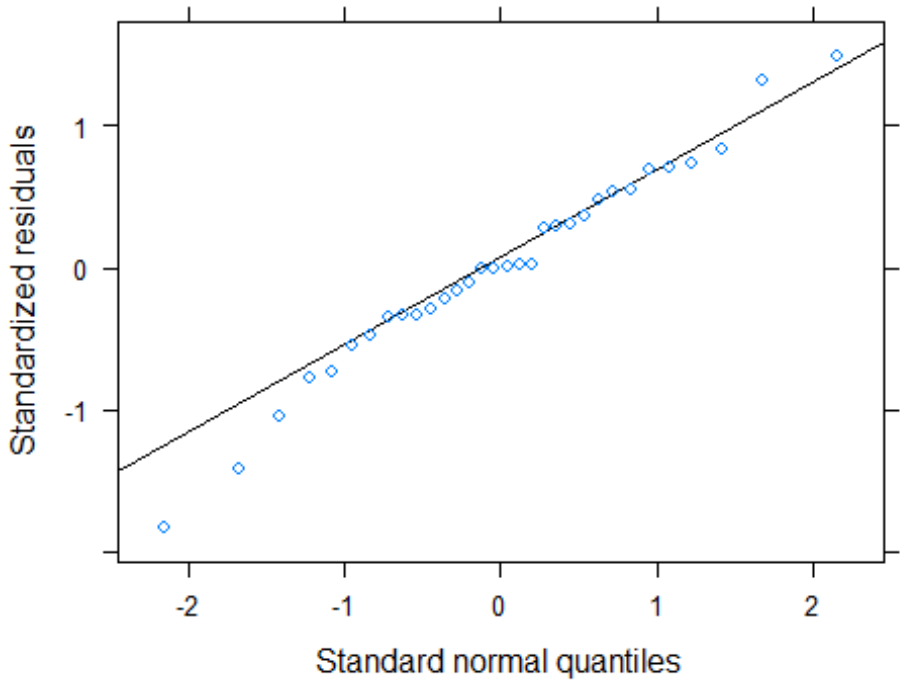
```
confint.merMod(modelP40,level=0.95,method="Wald")
```

```
##                            2.5 %      97.5 %
## .sig01                        NA          NA
## .sig02                        NA          NA
## .sigma                        NA          NA
## (Intercept)         17.8906817 18.65985511
## rescale(VAP)        -0.6517484  0.29781817
## rescale(SpermCount) -0.5066304  0.32858434
## StatusS              0.0281200  0.46095429
## StageB              -0.2393140  0.08068657
```

```
plot(modelP40, results="hide", fig.show='hide')#Visual Check Variance assumpt
ion
```




```
qqmath(modelP40)#Visual Check Normality assumption
```




```
shapiro.test(resid(modelP40))#Test Check Normality assumption
```

```
##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP40)
## W = 0.98185, p-value = 0.8512
```

```
rand(modelP40)#Test for significance of random predictor male ID
```

```
## Analysis of Random effects Table:
##          Chi.sq Chi.DF p.value
## MaleID 2.18e+00      1     0.1
## Week   1.10e-13      1     1.0
```

```
proteins[[41]]
```

```
## [1] "B5DGT2_SALSA"
```

```
modelP41<-lmer(B5DGT2_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status + S
tage +
                 (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats)
summary(modelP41)
```

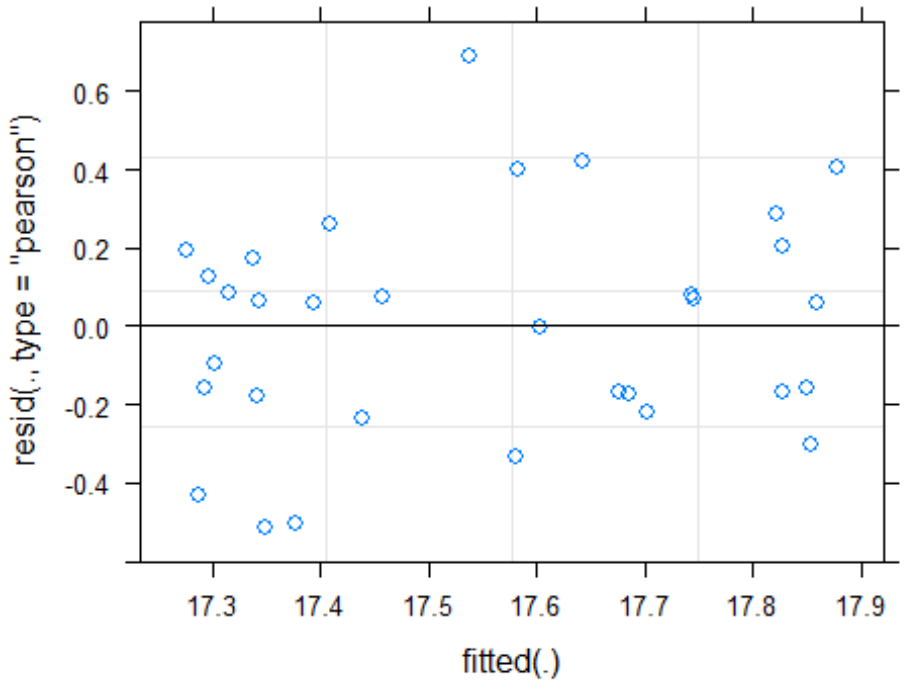
```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: B5DGT2_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
##     Stage + (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 27.6
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.6054 -0.5400  0.1861  0.5509  2.1502
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.010950 0.10464
##  Week     (Intercept) 0.005264 0.07255
##  Residual             0.103684 0.32200
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error       df t value Pr(>|t|)
## (Intercept)         17.54482    0.21106 20.42500  83.125  < 2e-16 ***
## rescale(VAP)        -0.08113    0.26627 19.10300  -0.305  0.76389
## rescale(SpermCount) -0.32020    0.26088 24.72500  -1.227  0.23123
## StatusS              0.42254    0.12634 23.46700   3.344  0.00276 **
## StageB              -0.01681    0.11713 11.66600  -0.144  0.88833
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
```

```
## Correlation of Fixed Effects:
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.789
## rscl(SprmC) -0.575  0.295
## StatusS     -0.051 -0.177 -0.239
## StageB      -0.135 -0.092 -0.193  0.128
```

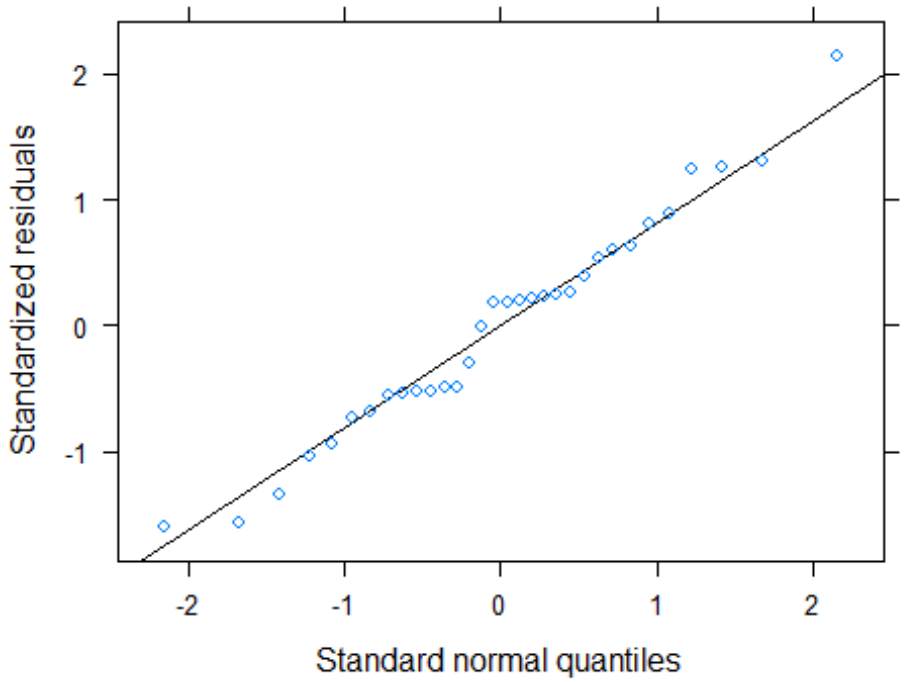
```
confint.merMod(modelP41,level=0.95,method="Wald")
```

```
##                            2.5 %     97.5 %
## .sig01                        NA         NA
## .sig02                        NA         NA
## .sigma                        NA         NA
## (Intercept)          17.1311366 17.9584946
## rescale(VAP)         -0.6030160  0.4407517
## rescale(SpermCount) -0.8315192  0.1911094
## StatusS               0.1749096  0.6701717
## StageB               -0.2463773  0.2127584
```

```
plot(modelP41, results="hide", fig.show='hide')#Visual Check Variance assumption
```




```
qqmath(modelP41)#Visual Check Normality assumption
```



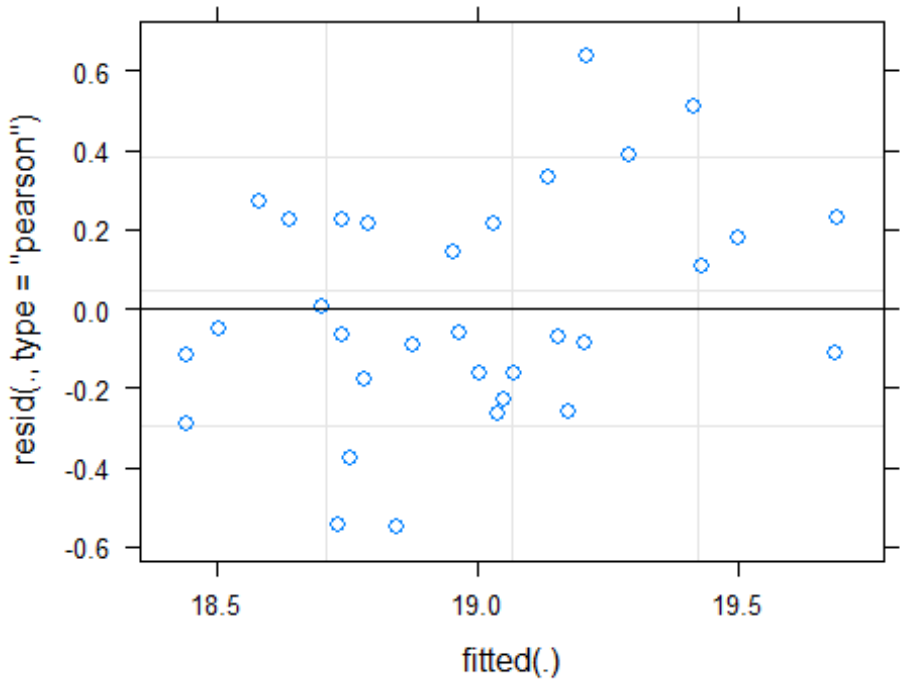

```
shapiro.test(resid(modelP41))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP41)
## W = 0.97744, p-value = 0.7224

rand(modelP41)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID 0.0744      1     0.8
## Week   0.1045      1     0.7

proteins[[44]]

## [1] "B5DGU8_SALSA"

modelP44<-lmer(B5DGU8_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status + S
tage +
                 (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats)
summary(modelP44)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: B5DGU8_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
##     Stage + (1 | MaleID) + (1 | Week)
```

```
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 41.1
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.5482 -0.4696 -0.1828  0.6105  1.7922
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.05393  0.2322
##  Week     (Intercept) 0.04455  0.2111
##  Residual             0.12742  0.3570
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error      df t value Pr(>|t|)
## (Intercept)          18.6286     0.2905 19.3880  64.119   <2e-16 ***
## rescale(VAP)          0.9398     0.3593 25.6790   2.616   0.0147 *
## rescale(SpermCount)   0.3366     0.3157 21.0150   1.066   0.2984
## StatusS              -0.4848     0.1560 21.6010  -3.108   0.0052 **
## StageB               -0.1763     0.1310 11.7750  -1.346   0.2038
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.783
## rscl(SprmC) -0.557  0.345
## StatusS     -0.058 -0.146 -0.239
## StageB      -0.097 -0.095 -0.193  0.149
```

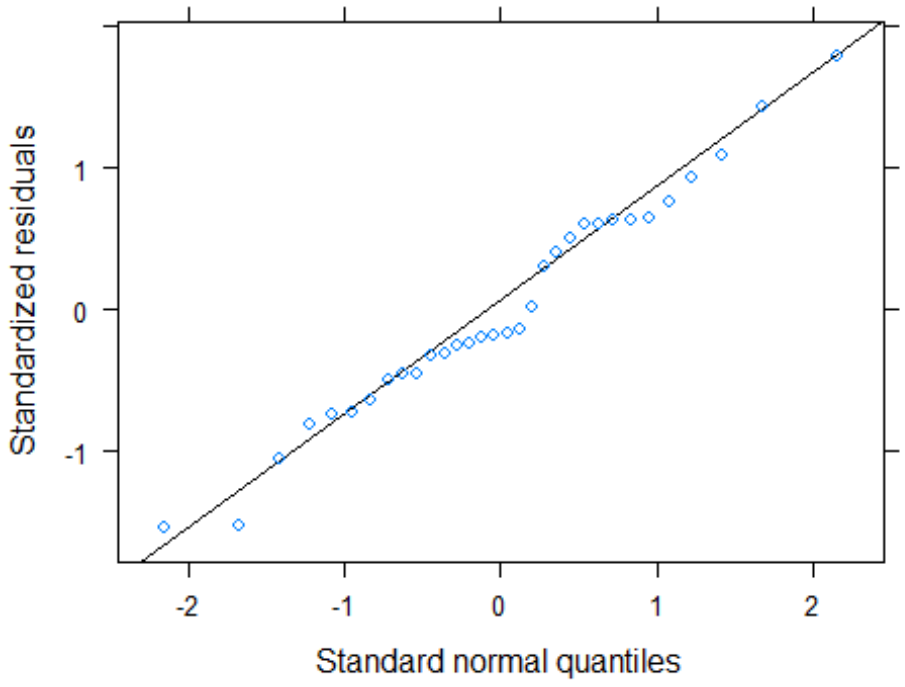
```
confint.merMod(modelP44,level=0.95,method="Wald")
```

```
##                            2.5 %      97.5 %
## .sig01                        NA          NA
## .sig02                        NA          NA
## .sigma                        NA          NA
## (Intercept)          18.0591644 19.19802391
## rescale(VAP)          0.2356462  1.64396083
## rescale(SpermCount) -0.2820670  0.95526074
## StatusS             -0.7904590 -0.17905749
## StageB              -0.4331273  0.08049056
```

```
plot(modelP44, results="hide", fig.show='hide')#Visual Check Variance assumpt
ion
```




```
qqmath(modelP44)#Visual Check Normality assumption
```




```
shapiro.test(resid(modelP44))#Test Check Normality assumption
```

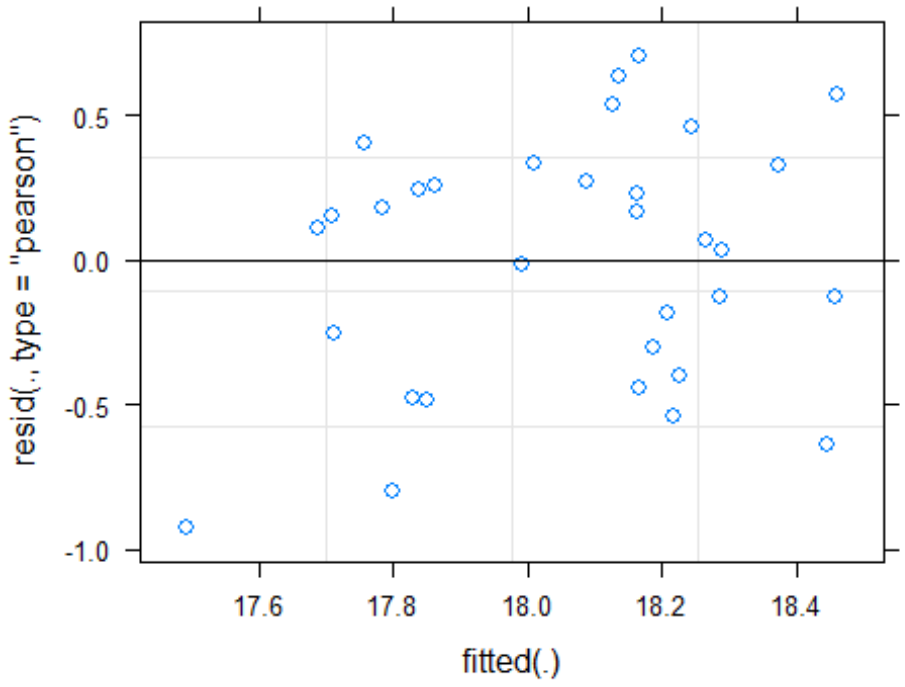
```
##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP44)
## W = 0.97742, p-value = 0.7218

rand(modelP44)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID  0.857      1     0.4
## Week    0.516      1     0.5

proteins[[66]]

## [1] "B5X1H4_SALSA"

modelP66<-lmer(B5X1H4_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status + S
tage +
                 (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats)
summary(modelP66)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: B5X1H4_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
##     Stage + (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 49.9
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.9043 -0.6691  0.1891  0.5864  1.4543
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.03309  0.1819
##  Week     (Intercept) 0.00000  0.0000
##  Residual             0.23703  0.4869
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error      df t value Pr(>|t|)
## (Intercept)          18.4231     0.3116 25.2800  59.133   <2e-16 ***
## rescale(VAP)         -0.2496     0.3908 24.6870  -0.639   0.5289
## rescale(SpermCount)   0.1385     0.3960 25.5870   0.350   0.7295
## StatusS              -0.4065     0.1936 26.8490  -2.099   0.0453 *
## StageB               -0.1975     0.1772 13.9930  -1.115   0.2837
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
```

```
## Correlation of Fixed Effects:
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.787
## rscl(SprmC) -0.581  0.287
## StatusS     -0.059 -0.178 -0.239
## StageB      -0.139 -0.094 -0.191  0.130
```

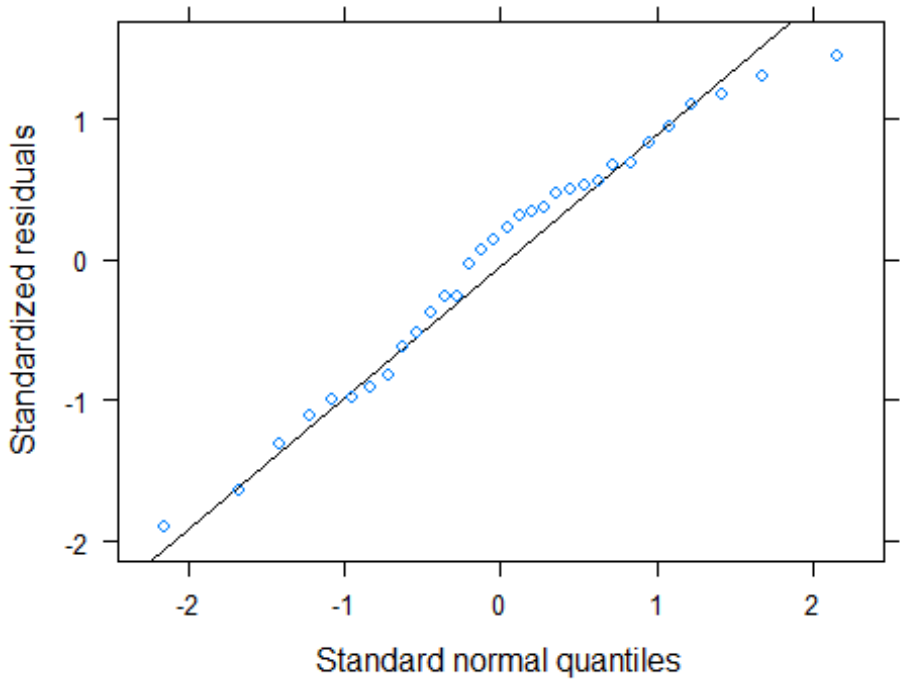
```
confint.merMod(modelP66,level=0.95,method="Wald")
```

```
##                            2.5 %      97.5 %
## .sig01                        NA          NA
## .sig02                        NA          NA
## .sigma                        NA          NA
## (Intercept)          17.8124849 19.03374647
## rescale(VAP)         -1.0155611  0.51632658
## rescale(SpermCount) -0.6377106  0.91463699
## StatusS              -0.7859605 -0.02696494
## StageB               -0.5447490  0.14972286
```

```
plot(modelP66, results="hide", fig.show='hide')#Visual Check Variance assumpt
ion
```




```
qqmath(modelP66)#Visual Check Normality assumption
```



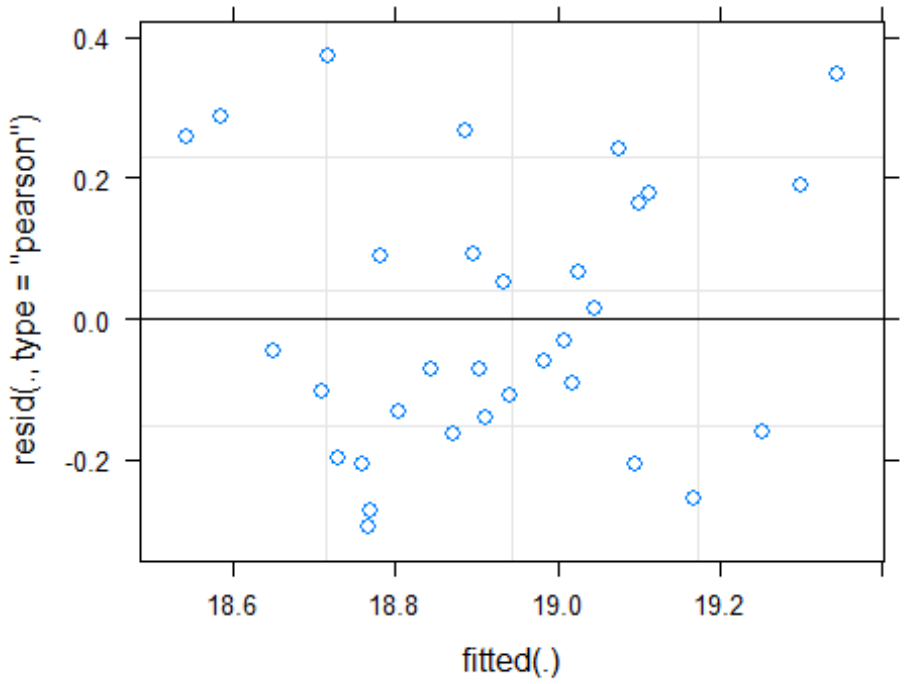

```
shapiro.test(resid(modelP66))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP66)
## W = 0.96911, p-value = 0.4752

rand(modelP66)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##          Chi.sq Chi.DF p.value
## MaleID 1.88e-01      1     0.7
## Week   2.13e-14      1     1.0

proteins[[73]]

## [1] "B5X1X1_SALSA"

modelP73<-lmer(B5X1X1_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status + S
tage +
                 (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats)
summary(modelP73)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: B5X1X1_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
##     Stage + (1 | MaleID) + (1 | Week)
```

```
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 4.9
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.3864 -0.6774 -0.2505  0.7819  1.7510
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.000000 0.0000
##  Week     (Intercept) 0.008083 0.0899
##  Residual             0.045880 0.2142
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error       df t value Pr(>|t|)
## (Intercept)         19.39420    0.14323 21.49600 135.407  < 2e-16 ***
## rescale(VAP)        -0.38471    0.18049 23.87100  -2.131  0.04355 *
## rescale(SpermCount) -0.23639    0.16883 24.70600  -1.400  0.17390
## StatusS             -0.25942    0.07977 23.31600  -3.252  0.00347 **
## StageB              -0.09142    0.07771 23.10700  -1.176  0.25139
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.780
## rscl(SprmC) -0.561  0.302
## StatusS     -0.035 -0.180 -0.237
## StageB      -0.132 -0.088 -0.195  0.118
```

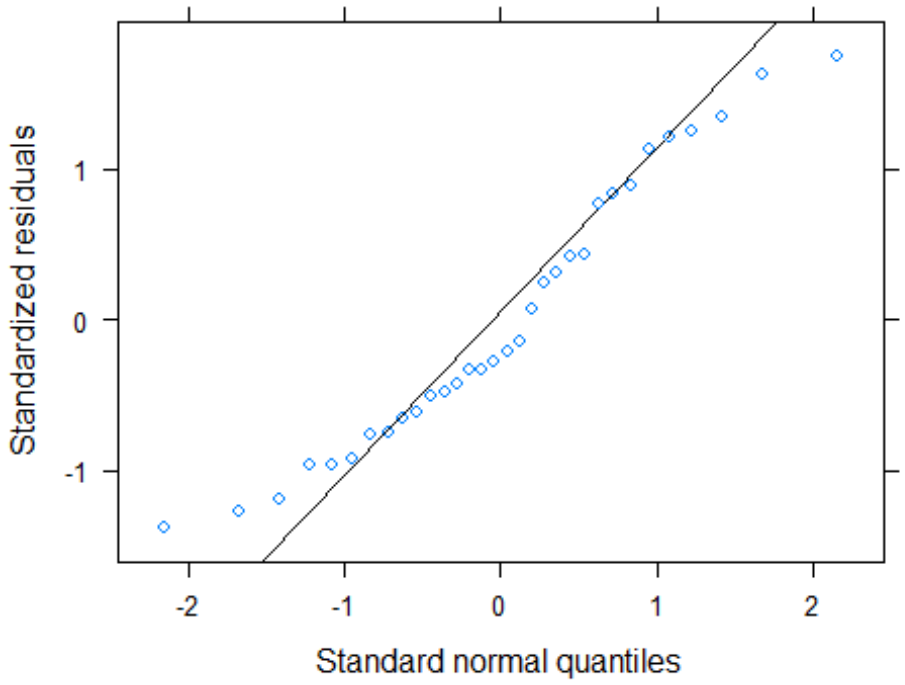
```
confint.merMod(modelP73,level=0.95,method="Wald")
```

```
##                            2.5 %      97.5 %
## .sig01                        NA          NA
## .sig02                        NA          NA
## .sigma                        NA          NA
## (Intercept)         19.1134769 19.67492495
## rescale(VAP)        -0.7384616 -0.03095210
## rescale(SpermCount) -0.5672986  0.09451627
## StatusS             -0.4157656 -0.10306806
## StageB              -0.2437335  0.06088745
```

```
plot(modelP73, results="hide", fig.show='hide')#Visual Check Variance assumpt
ion
```




```
qqmath(modelP73)#Visual Check Normality assumption
```




```
shapiro.test(resid(modelP73))#Test Check Normality assumption
```

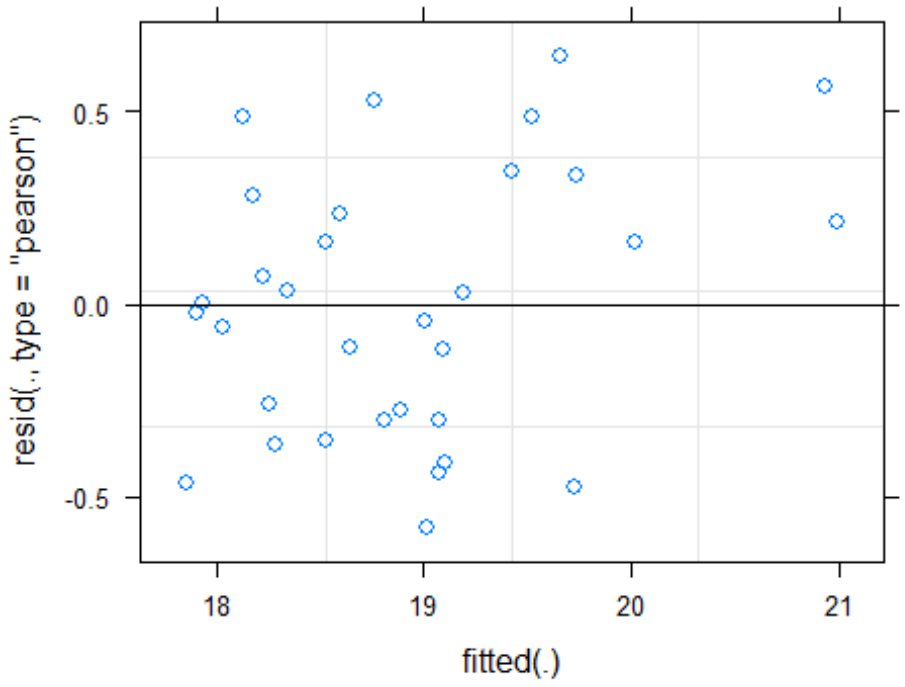
```
##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP73)
## W = 0.94871, p-value = 0.1324

rand(modelP73)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##          Chi.sq Chi.DF p.value
## MaleID 7.11e-15      1     1.0
## Week   7.93e-01      1     0.4

proteins[[99]]

## [1] "B5X4I3_SALSA"

modelP99<-lmer(B5X4I3_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status + S
tage +
                 (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats)
summary(modelP99)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: B5X4I3_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
##     Stage + (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 68.1
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.20322 -0.61888 -0.02196  0.50992  1.33541
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.2754   0.5248
##  Week     (Intercept) 0.3414   0.5843
##  Residual             0.2341   0.4839
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error       df t value Pr(>|t|)
## (Intercept)         19.21422    0.51538 18.25300  37.281   <2e-16 ***
## rescale(VAP)        -0.14994    0.57634 23.97300  -0.260   0.7970
## rescale(SpermCount) -1.06278    0.46883 19.35400  -2.267   0.0350 *
## StatusS              0.55282    0.23735 21.36900   2.329   0.0297 *
## StageB              -0.08335    0.17943 12.74600  -0.465   0.6501
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
```

```
## Correlation of Fixed Effects:
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.722
## rscl(SprmC) -0.515  0.398
## StatusS     -0.075 -0.107 -0.227
## StageB      -0.069 -0.097 -0.187  0.176
```

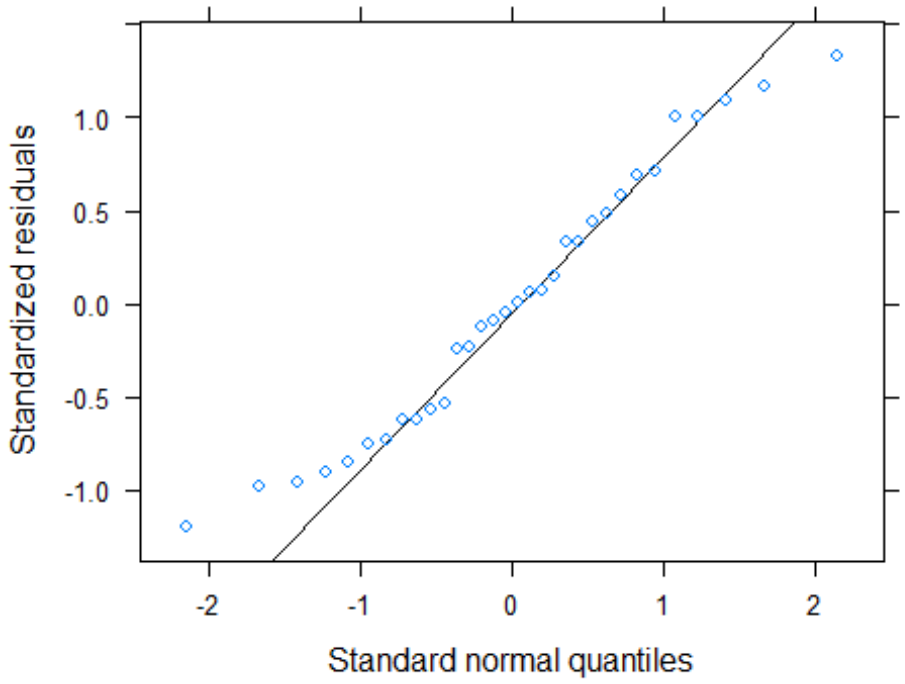
```
confint.merMod(modelP99,level=0.95,method="Wald")
```

```
##                             2.5 %     97.5 %
## .sig01                         NA         NA
## .sig02                         NA         NA
## .sigma                         NA         NA
## (Intercept)          18.20408426 20.2243475
## rescale(VAP)         -1.27955222  0.9796761
## rescale(SpermCount) -1.98166816 -0.1438967
## StatusS               0.08762539  1.0180078
## StageB               -0.43502339  0.2683287
```

```
plot(modelP99, results="hide", fig.show='hide')#Visual Check Variance assumpt
ion
```




```
qqmath(modelP99)#Visual Check Normality assumption
```



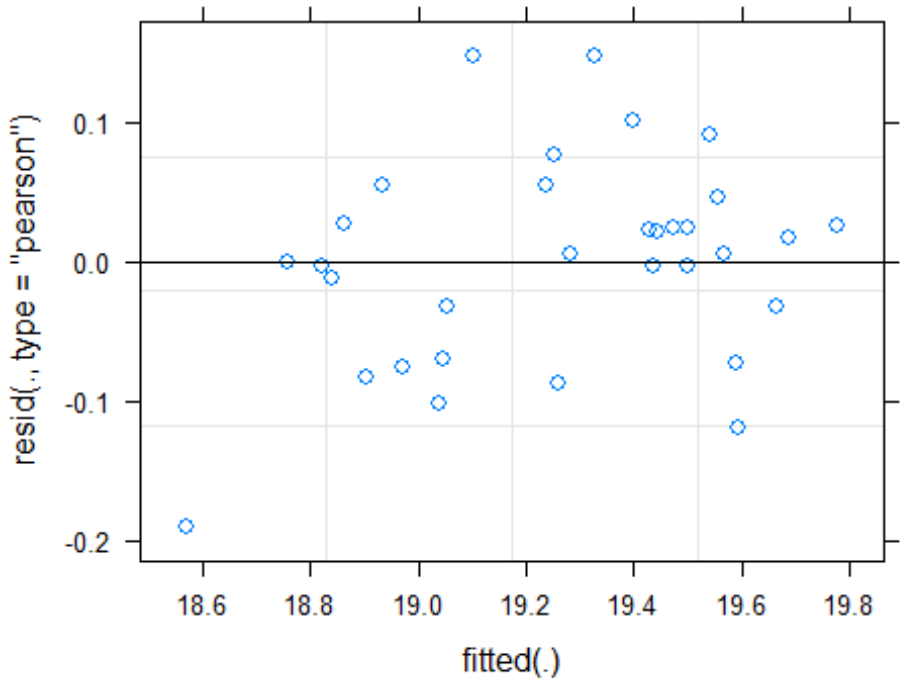

```
shapiro.test(resid(modelP99))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP99)
## W = 0.96194, p-value = 0.3101

rand(modelP99)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID   3.87      1    0.05 *
## Week     3.53      1    0.06 .
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

proteins[[153]]

## [1] "B5XBK1_SALSA"

modelP153<-lmer(B5XBK1_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
Stage +
                  (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats)
summary(modelP153)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
```

```
## Formula: B5XBK1_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
##     Stage + (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 12.1
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.61072 -0.35553  0.04323  0.26517  1.24777
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.11547  0.3398
##  Week     (Intercept) 0.00000  0.0000
##  Residual             0.01409  0.1187
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error       df t value Pr(>|t|)
## (Intercept)         18.85757    0.15006 25.15000 125.669  < 2e-16 ***
## rescale(VAP)         0.46759    0.16502 15.77300   2.833  0.01211 *
## rescale(SpermCount) -0.08698    0.12910 12.80000  -0.674  0.51245
## StatusS              0.18967    0.06786 12.77000   2.795  0.01539 *
## StageB               0.19284    0.04467 10.72600   4.318  0.00129 **
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.752
## rscl(SprmC) -0.549  0.460
## StatusS     -0.126 -0.048 -0.188
## StageB      -0.061 -0.093 -0.169  0.216
```

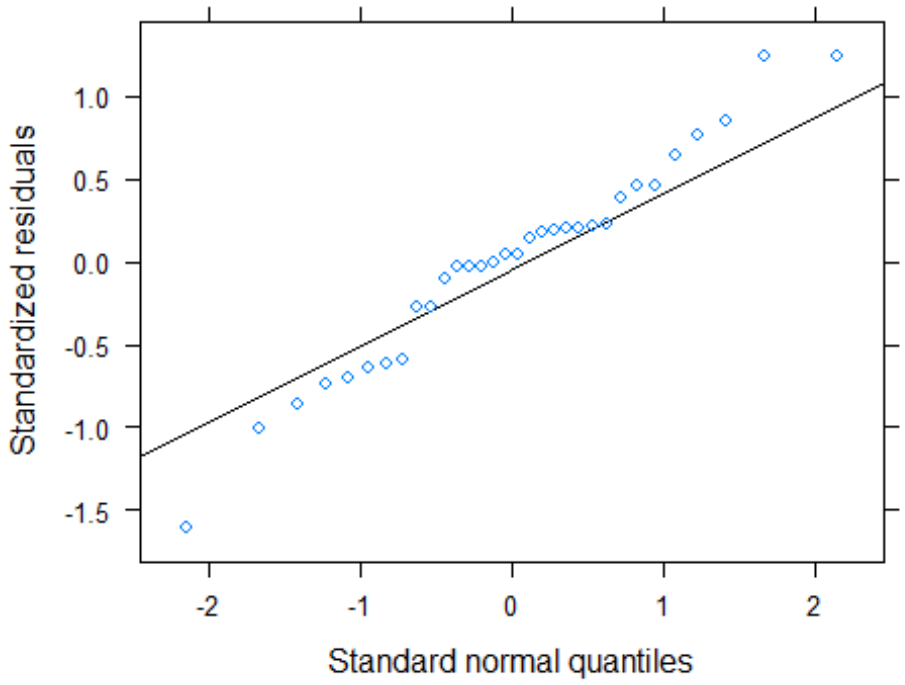
```
confint.merMod(modelP153,level=0.95,method="Wald")
```

```
##                            2.5 %     97.5 %
## .sig01                        NA         NA
## .sig02                        NA         NA
## .sigma                        NA         NA
## (Intercept)         18.56346548 19.1516804
## rescale(VAP)         0.14415126  0.7910317
## rescale(SpermCount) -0.34000911  0.1660465
## StatusS              0.05667313  0.3226642
## StageB               0.10530222  0.2803863
```

```
plot(modelP153, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```




```
qqmath(modelP153)#Visual Check Normality assumption
```




```
shapiro.test(resid(modelP153))#Test Check Normality assumption
```

```
##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP153)
## W = 0.97234, p-value = 0.5664
```

```
rand(modelP153)#Test for significance of random predictor male ID
```

```
## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID   13.6      1   2e-04 ***
## Week      0.0      1       1
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
proteins[[171]]
```

```
## [1] "B5XDG6_SALSA"
```

```
modelP171<-lmer(B5XDG6_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
Stage +
                  (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats
)
summary(modelP171)
```

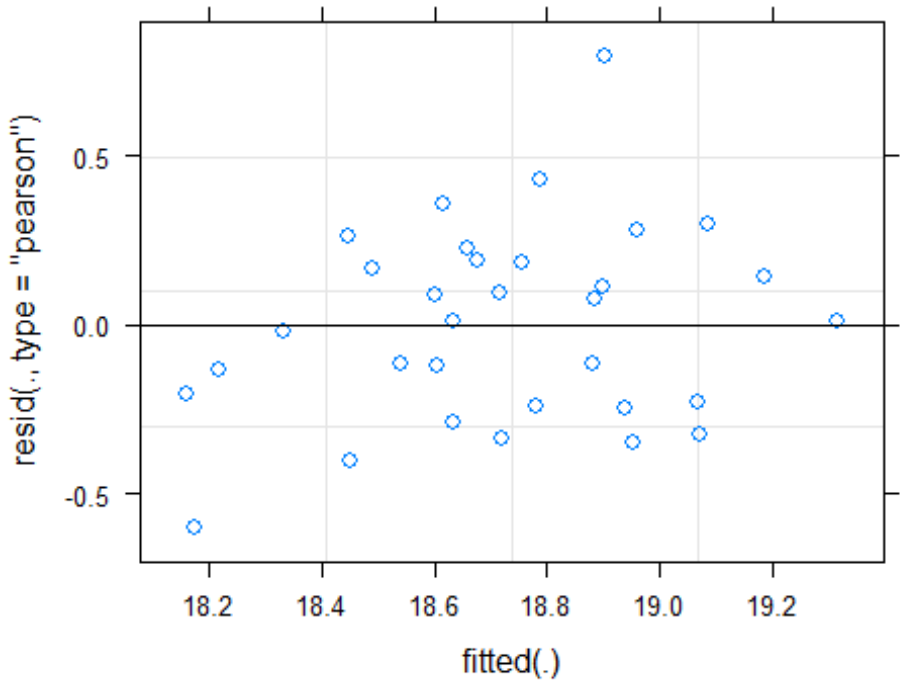
```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: B5XDG6_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
##     Stage + (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 34.2
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.74464 -0.67394  0.02666  0.53763  2.31179
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.0347   0.1863
##  Week     (Intercept) 0.0000   0.0000
##  Residual             0.1194   0.3455
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error      df t value Pr(>|t|)
## (Intercept)          18.8845     0.2362 25.9280  79.958  < 2e-16 ***
## rescale(VAP)          0.1218     0.2966 25.8830   0.411  0.68469
## rescale(SpermCount)   0.3621     0.2924 24.7850   1.238  0.22714
## StatusS              -0.4311     0.1451 26.9550  -2.971  0.00618 **
## StageB               -0.3986     0.1263 14.6390  -3.157  0.00668 **
```

```
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.795
## rscl(SprmC) -0.580  0.302
## StatusS     -0.065 -0.168 -0.240
## StageB      -0.125 -0.096 -0.191  0.141
```

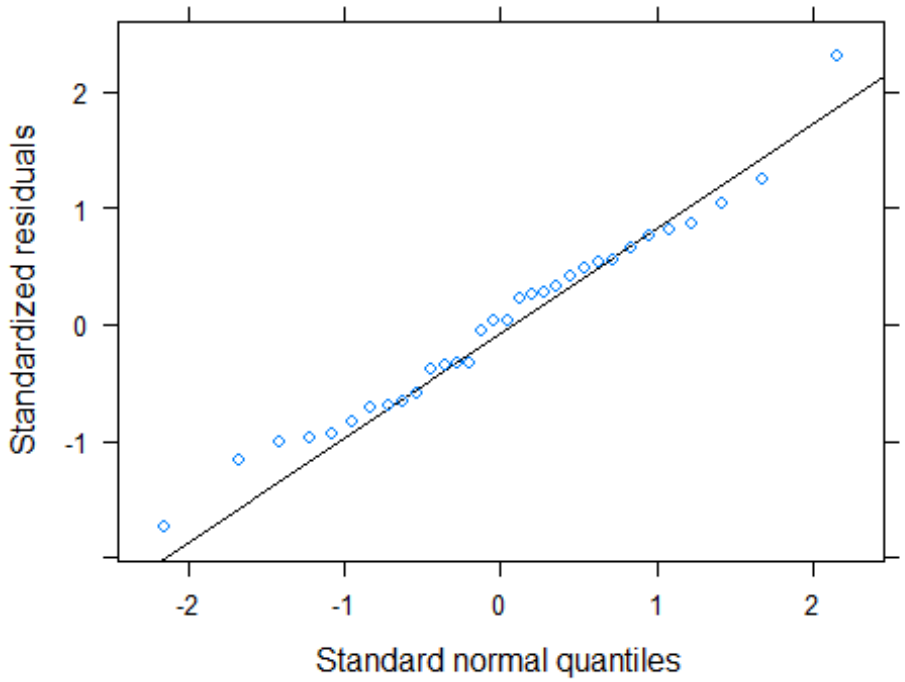
```
confint.merMod(modelP171,level=0.95,method="Wald")
```

```
##                            2.5 %     97.5 %
## .sig01                        NA         NA
## .sig02                        NA         NA
## .sigma                        NA         NA
## (Intercept)          18.4216098 19.3474208
## rescale(VAP)         -0.4595625  0.7031950
## rescale(SpermCount) -0.2109686  0.9352388
## StatusS              -0.7155487 -0.1466941
## StageB               -0.6460260 -0.1510964
```

```
plot(modelP171, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```




```
qqmath(modelP171)#Visual Check Normality assumption
```



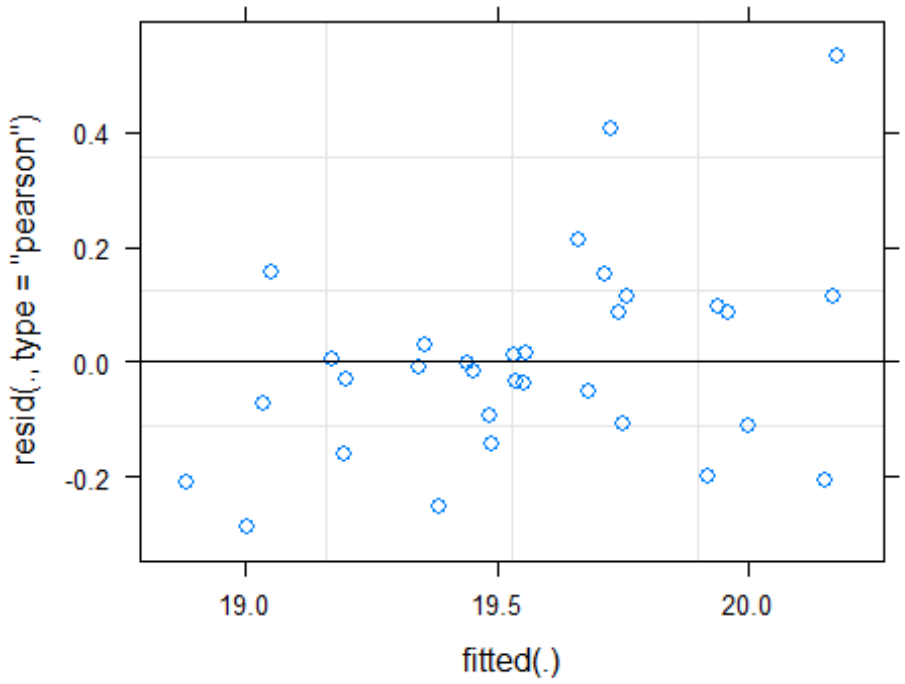

```
shapiro.test(resid(modelP171))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP171)
## W = 0.9789, p-value = 0.7669

rand(modelP171)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID  0.738      1     0.4
## Week    0.000      1     1.0

proteins[[191]]

## [1] "B5XG37_SALSA"

modelP191<-lmer(B5XG37_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
Stage +
                  (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats
)
summary(modelP191)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: B5XG37_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
```

```
##     Stage + (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 33
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.15031 -0.43284 -0.05135  0.35291  2.13245
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.11015  0.3319
##  Week     (Intercept) 0.01581  0.1257
##  Residual             0.06266  0.2503
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error       df t value Pr(>|t|)
## (Intercept)         19.72099    0.24268 20.76800  81.262  < 2e-16 ***
## rescale(VAP)        -0.20028    0.29397 22.71700  -0.681  0.50257
## rescale(SpermCount) -0.12271    0.24762 18.33600  -0.496  0.62609
## StatusS              0.27960    0.12800 19.68700   2.184  0.04121 *
## StageB              -0.28570    0.09311 12.52200  -3.068  0.00933 **
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.797
## rscl(SprmC) -0.571  0.395
## StatusS     -0.097 -0.103 -0.223
## StageB      -0.078 -0.098 -0.182  0.186
```

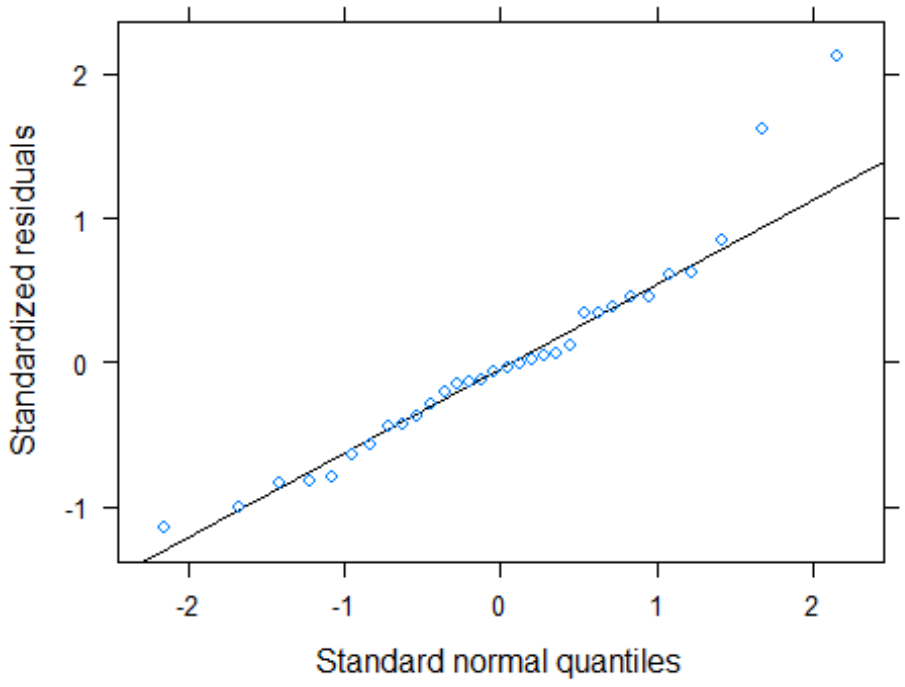
```
confint.merMod(modelP191,level=0.95,method="Wald")
```

```
##                            2.5 %     97.5 %
## .sig01                        NA         NA
## .sig02                        NA         NA
## .sigma                        NA         NA
## (Intercept)         19.24533240 20.1966395
## rescale(VAP)        -0.77644338  0.3758872
## rescale(SpermCount) -0.60802496  0.3626096
## StatusS              0.02872834  0.5304675
## StageB              -0.46819700 -0.1032057
```

```
plot(modelP191, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```




```
qqmath(modelP191)#Visual Check Normality assumption
```




```
shapiro.test(resid(modelP191))#Test Check Normality assumption
```

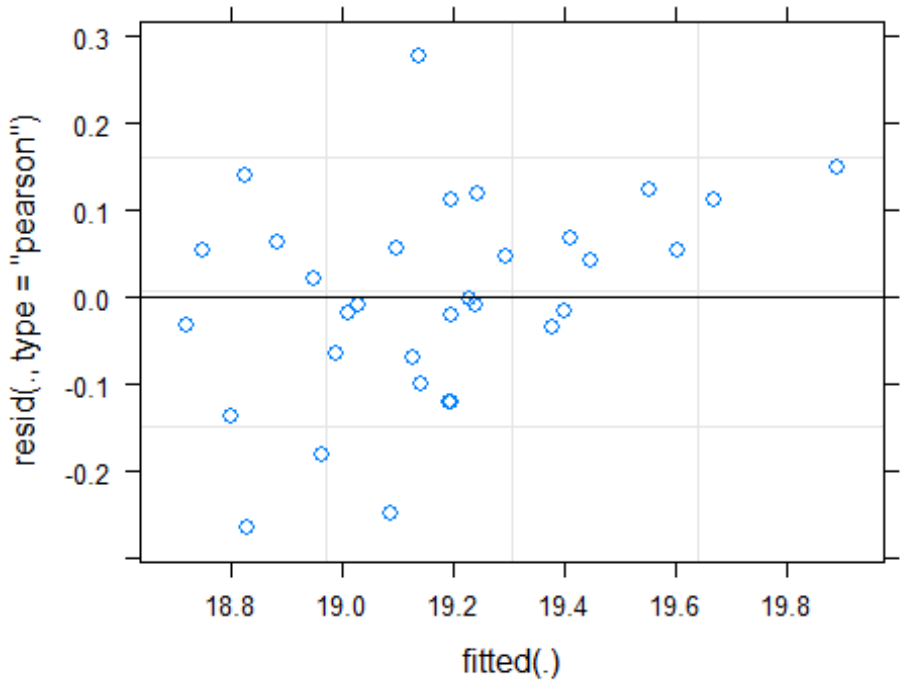
```
##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP191)
## W = 0.9337, p-value = 0.04971

rand(modelP191)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID  5.973      1    0.01 *
## Week    0.165      1    0.68
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

proteins[[192]]

## [1] "B5XG91_SALSA"

modelP192<-lmer(B5XG91_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
Stage +
                  (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats
)
summary(modelP192)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: B5XG91_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
##     Stage + (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 18.2
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.52119 -0.37485 -0.02977  0.36952  1.59460
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.08652  0.2941
##  Week     (Intercept) 0.00000  0.0000
##  Residual             0.03037  0.1743
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error       df t value Pr(>|t|)
## (Intercept)         19.41944    0.17731 26.19300 109.525   <2e-16 ***
## rescale(VAP)         0.10203    0.21328 22.32600   0.478   0.6370
## rescale(SpermCount) -0.37105    0.17802 15.54100  -2.084   0.0540 .
## StatusS             -0.25357    0.09319 16.39000  -2.721   0.0149 *
## StageB              -0.13599    0.06510 10.99900  -2.089   0.0608 .
```

```
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.805
## rscl(SprmC) -0.580  0.411
## StatusS     -0.112 -0.088 -0.214
## StageB      -0.074 -0.098 -0.178  0.198
```

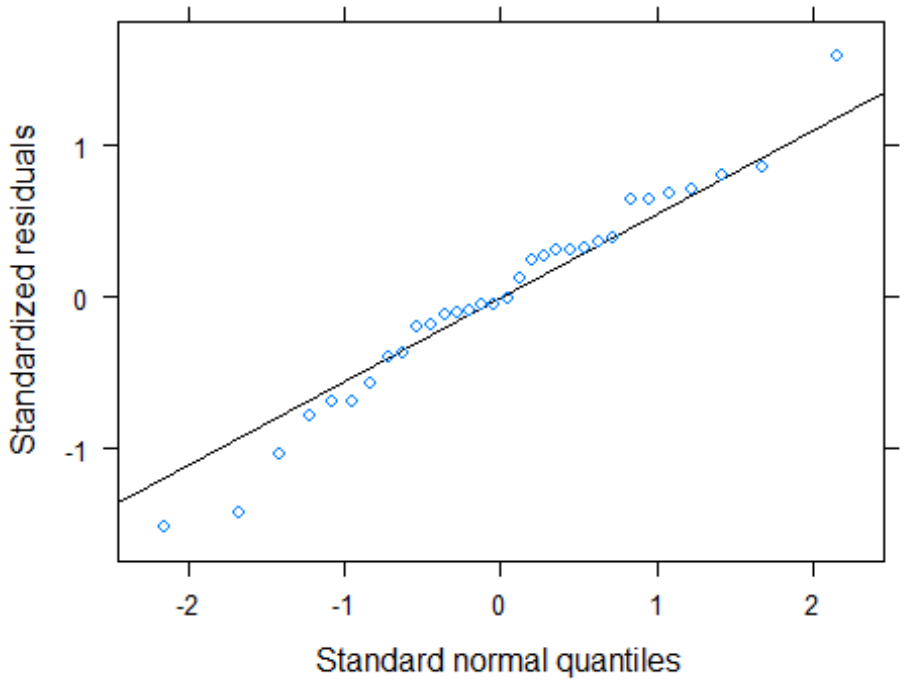
```
confint.merMod(modelP192,level=0.95,method="Wald")
```

```
##                            2.5 %       97.5 %
## .sig01                        NA           NA
## .sig02                        NA           NA
## .sigma                        NA           NA
## (Intercept)          19.0719296 19.766954993
## rescale(VAP)         -0.3159840  0.520038897
## rescale(SpermCount) -0.7199667 -0.022135425
## StatusS              -0.4362217 -0.070909424
## StageB               -0.2635763 -0.008394785
```

```
plot(modelP192, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```




```
qqmath(modelP192)#Visual Check Normality assumption
```



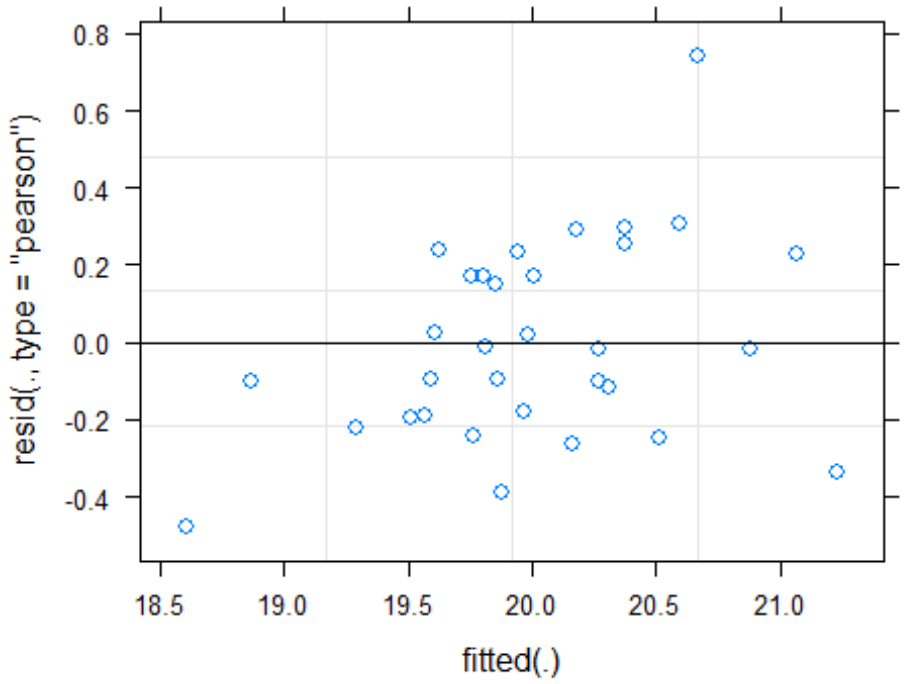

```
shapiro.test(resid(modelP192))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP192)
## W = 0.97675, p-value = 0.701

rand(modelP192)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##          Chi.sq Chi.DF p.value
## MaleID 7.32e+00      1   0.007 **
## Week   4.97e-14      1   1.000
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

proteins[[198]]

## [1] "B8R4G1_ONCTS"

modelP198<-lmer(B8R4G1_ONCTS ~ rescale(VAP) + rescale(SpermCount) + Status +
Stage +
                  (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats
)
summary(modelP198)
```

```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: B8R4G1_ONCTS ~ rescale(VAP) + rescale(SpermCount) + Status +
##     Stage + (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 55.6
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.30573 -0.51940 -0.04235  0.50121  2.02158
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.2023   0.4497
##  Week     (Intercept) 0.2047   0.4524
##  Residual             0.1356   0.3683
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error      df t value Pr(>|t|)
## (Intercept)          19.5842     0.4049 15.5980  48.365   <2e-16 ***
## rescale(VAP)          0.7633     0.4504 22.5980   1.695   0.1039
## rescale(SpermCount)   0.8444     0.3637 17.7400   2.321   0.0324 *
## StatusS              -0.4550     0.1854 19.4180  -2.454   0.0237 *
## StageB               -0.1589     0.1369 11.8410  -1.161   0.2685
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.723
## rscl(SprmC) -0.517  0.408
## StatusS     -0.082 -0.099 -0.223
## StageB      -0.067 -0.097 -0.184  0.182
```

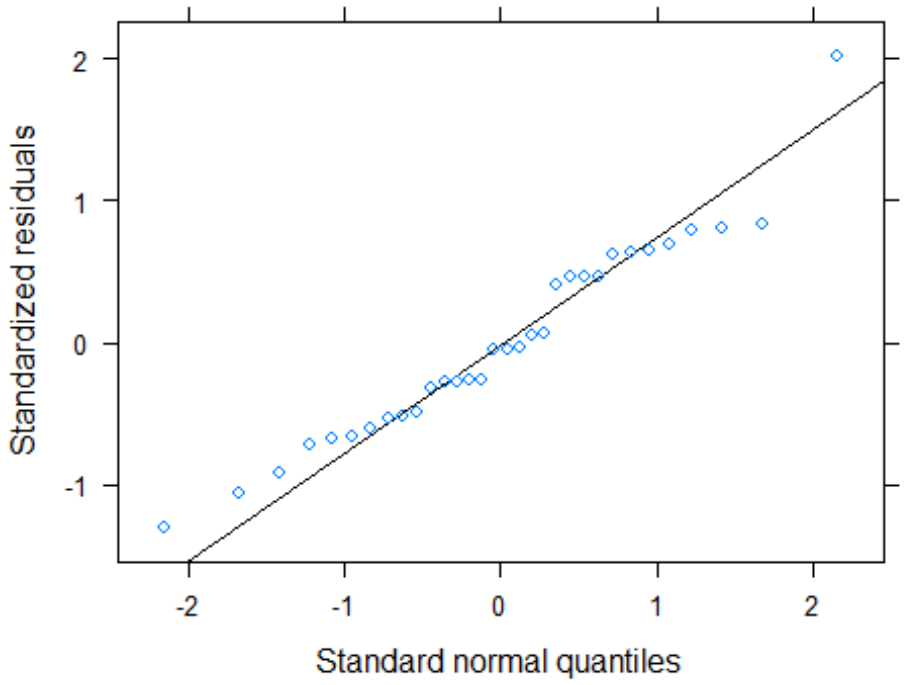
```
confint.merMod(modelP198,level=0.95,method="Wald")
```

```
##                            2.5 %      97.5 %
## .sig01                        NA          NA
## .sig02                        NA          NA
## .sigma                        NA          NA
## (Intercept)          18.7905350 20.37779547
## rescale(VAP)         -0.1194373  1.64605346
## rescale(SpermCount)   0.1314823  1.55727956
## StatusS              -0.8183559 -0.09162847
## StageB               -0.4271715  0.10936376
```

```
plot(modelP198, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```




```
qqmath(modelP198)#Visual Check Normality assumption
```




```
shapiro.test(resid(modelP198))#Test Check Normality assumption
```

```
##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP198)
## W = 0.95892, p-value = 0.2566
```

```
rand(modelP198)#Test for significance of random predictor male ID
```

```
## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID   4.09      1    0.04 *
## Week     1.49      1    0.22
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
proteins[[247]]
```

```
## [1] "C0HAB7_SALSA"
```

```
modelP247<-lmer(C0HAB7_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
Stage +
                  (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats
)
summary(modelP247)
```

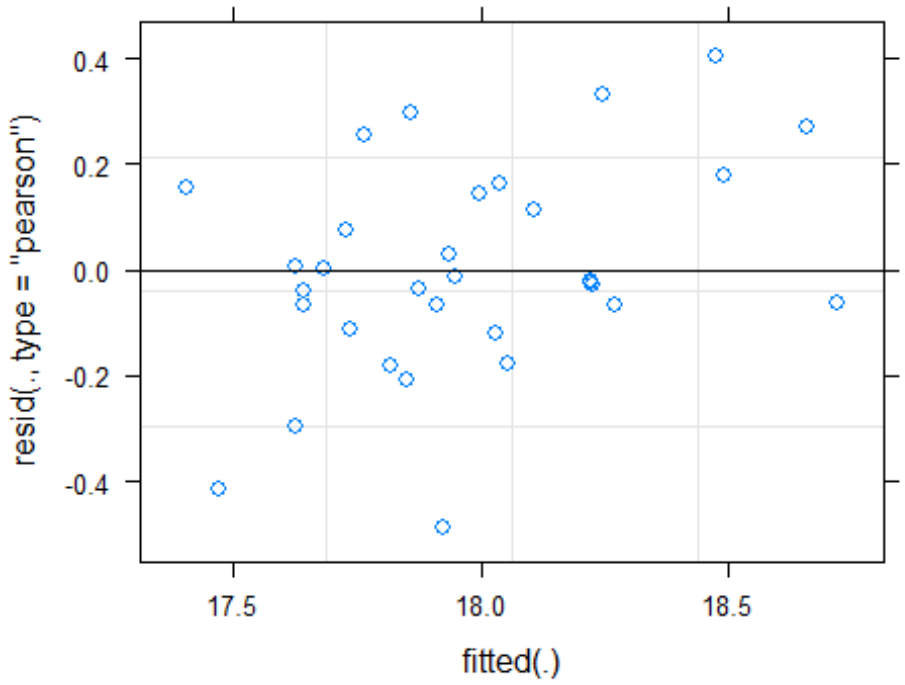
```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: C0HAB7_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
##     Stage + (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 32.4
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.78174 -0.28584 -0.08069  0.53189  1.47743
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.07084  0.2662
##  Week     (Intercept) 0.02590  0.1609
##  Residual             0.07549  0.2748
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error      df t value Pr(>|t|)
## (Intercept)          18.1515     0.2457 21.9550  73.863   <2e-16 ***
## rescale(VAP)          0.2521     0.3010 24.9080   0.837   0.4103
## rescale(SpermCount)  -0.1108     0.2586 19.6440  -0.429   0.6729
## StatusS              -0.3159     0.1312 21.8560  -2.409   0.0249 *
## StageB               -0.2759     0.1016 11.8080  -2.716   0.0190 *
```

```
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.791
## rscl(SprmC) -0.564  0.371
## StatusS     -0.078 -0.124 -0.233
## StageB      -0.085 -0.097 -0.188  0.169
```

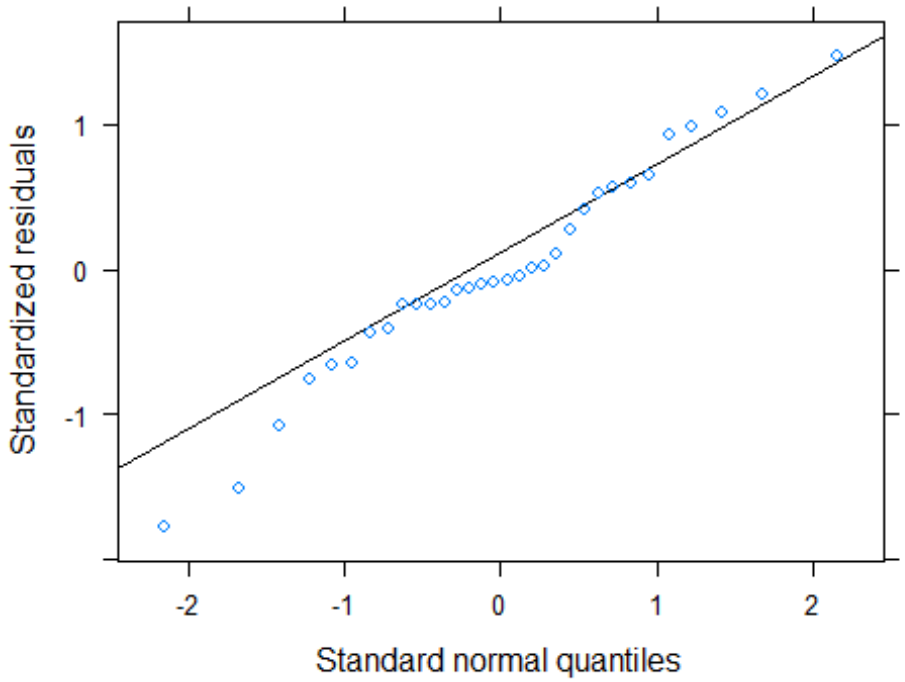
```
confint.merMod(modelP247,level=0.95,method="Wald")
```

```
##                            2.5 %      97.5 %
## .sig01                        NA          NA
## .sig02                        NA          NA
## .sigma                        NA          NA
## (Intercept)          17.6698458 18.63314626
## rescale(VAP)         -0.3379108  0.84210998
## rescale(SpermCount) -0.6176877  0.39599877
## StatusS              -0.5730007 -0.05887906
## StageB               -0.4750266 -0.07684577
```

```
plot(modelP247, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```




```
qqmath(modelP247)#Visual Check Normality assumption
```



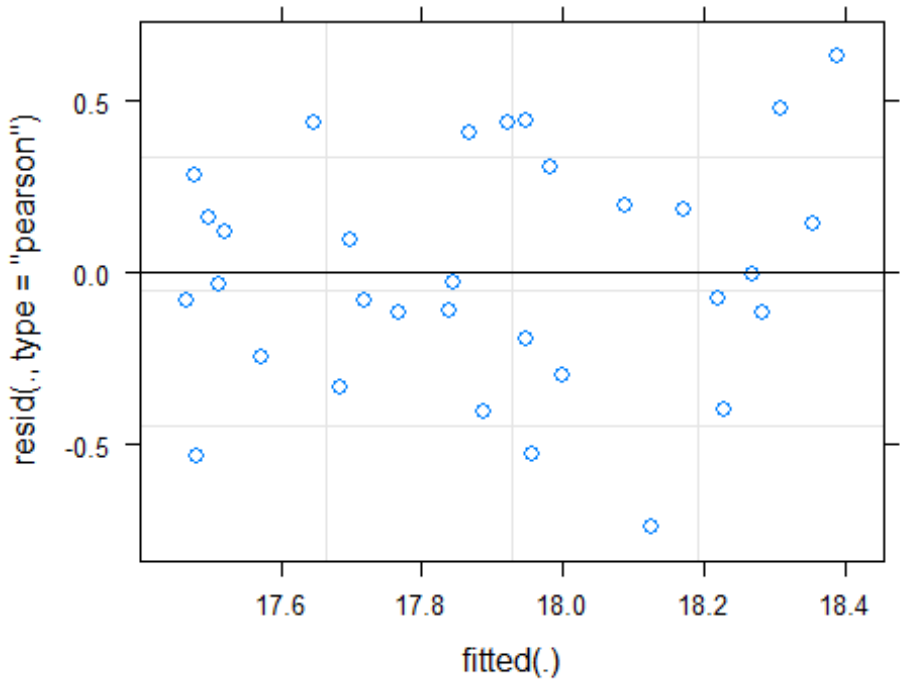

```
shapiro.test(resid(modelP247))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP247)
## W = 0.97407, p-value = 0.6183

rand(modelP247)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID  2.453      1     0.1
## Week    0.616      1     0.4

proteins[[248]]

## [1] "C0HAD5_SALSA"

modelP248<-lmer(C0HAD5_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
Stage +
                  (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats
)
summary(modelP248)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: C0HAD5_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
```

```
##     Stage + (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 35.7
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.96528 -0.54572 -0.08752  0.57465  1.67743
##
## Random effects:
##  Groups   Name        Variance  Std.Dev.
##  MaleID   (Intercept) 1.702e-02 1.304e-01
##  Week     (Intercept) 3.327e-19 5.768e-10
##  Residual             1.427e-01 3.778e-01
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error      df t value Pr(>|t|)
## (Intercept)          18.0948     0.2393 25.1950  75.617   <2e-16 ***
## rescale(VAP)         -0.1280     0.3000 24.5000  -0.427   0.6735
## rescale(SpermCount)  -0.2476     0.3053 25.7290  -0.811   0.4249
## StatusS              -0.3999     0.1489 26.7670  -2.685   0.0123 *
## StageB                0.3112     0.1374 14.0010   2.265   0.0399 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.786
## rscl(SprmC) -0.581  0.285
## StatusS     -0.058 -0.180 -0.238
## StageB      -0.141 -0.093 -0.191  0.128
```

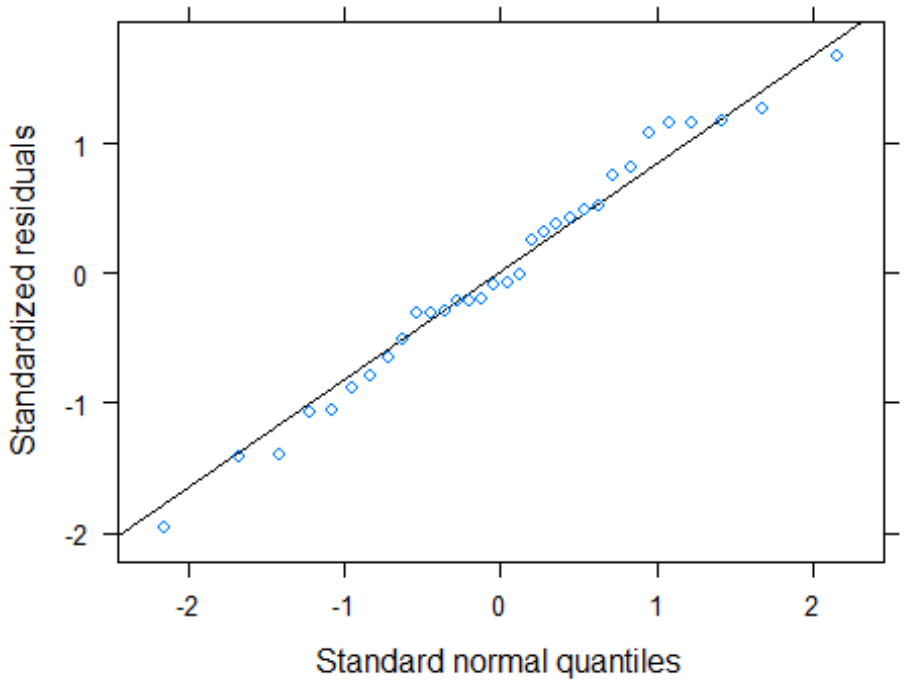
```
confint.merMod(modelP248,level=0.95,method="Wald")
```

```
##                            2.5 %     97.5 %
## .sig01                        NA         NA
## .sig02                        NA         NA
## .sigma                        NA         NA
## (Intercept)          17.6257511 18.5637753
## rescale(VAP)         -0.7160583  0.4601082
## rescale(SpermCount) -0.8460005  0.3508909
## StatusS              -0.6917812 -0.1079981
## StageB                0.0419033  0.5804178
```

```
plot(modelP248, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```




```
qqmath(modelP248)#Visual Check Normality assumption
```




```
shapiro.test(resid(modelP248))#Test Check Normality assumption
```

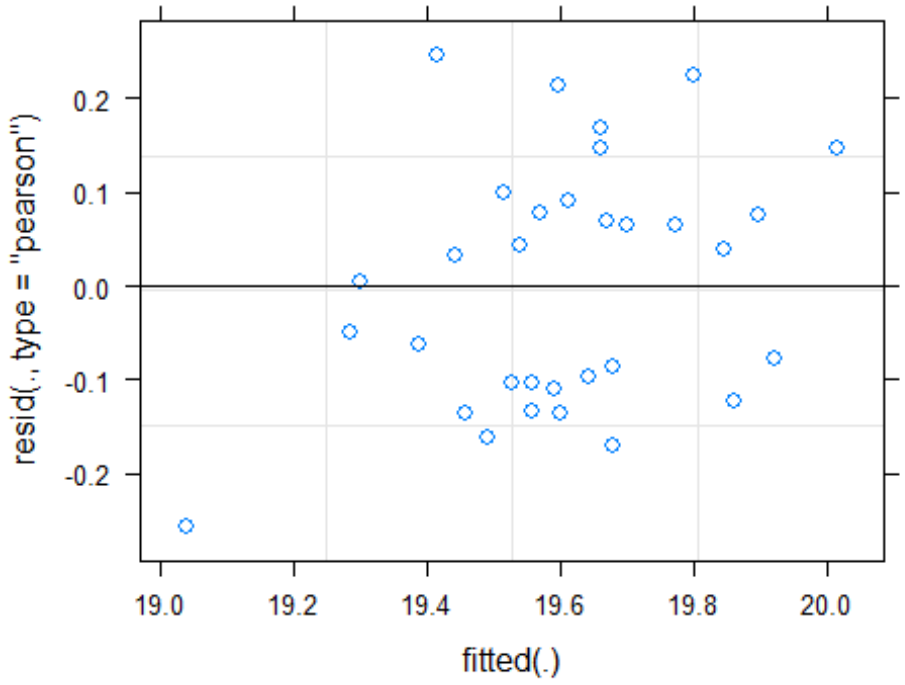
```
##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP248)
## W = 0.98208, p-value = 0.8572

rand(modelP248)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID  0.142      1     0.7
## Week    0.000      1     1.0

proteins[[262]]

## [1] "C0PUT9_SALSA"

modelP262<-lmer(C0PUT9_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
Stage +
                  (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats
)
summary(modelP262)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: C0PUT9_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
##     Stage + (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 4.4
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.5037 -0.6131  0.1086  0.4751  1.4416
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.01899  0.1378
##  Week     (Intercept) 0.01183  0.1087
##  Residual             0.02934  0.1713
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error       df t value Pr(>|t|)
## (Intercept)         19.70467    0.14779 21.99200 133.326  < 2e-16 ***
## rescale(VAP)         0.02886    0.18124 25.61200   0.159  0.87473
## rescale(SpermCount)  0.17173    0.15661 21.25800   1.097  0.28509
## StatusS             -0.19757    0.07839 23.50400  -2.520  0.01894 *
## StageB              -0.22433    0.06311 11.97500  -3.555  0.00398 **
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
##
## Correlation of Fixed Effects:
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.784
## rscl(SprmC) -0.557  0.360
## StatusS     -0.067 -0.134 -0.237
## StageB      -0.089 -0.097 -0.191  0.160
```

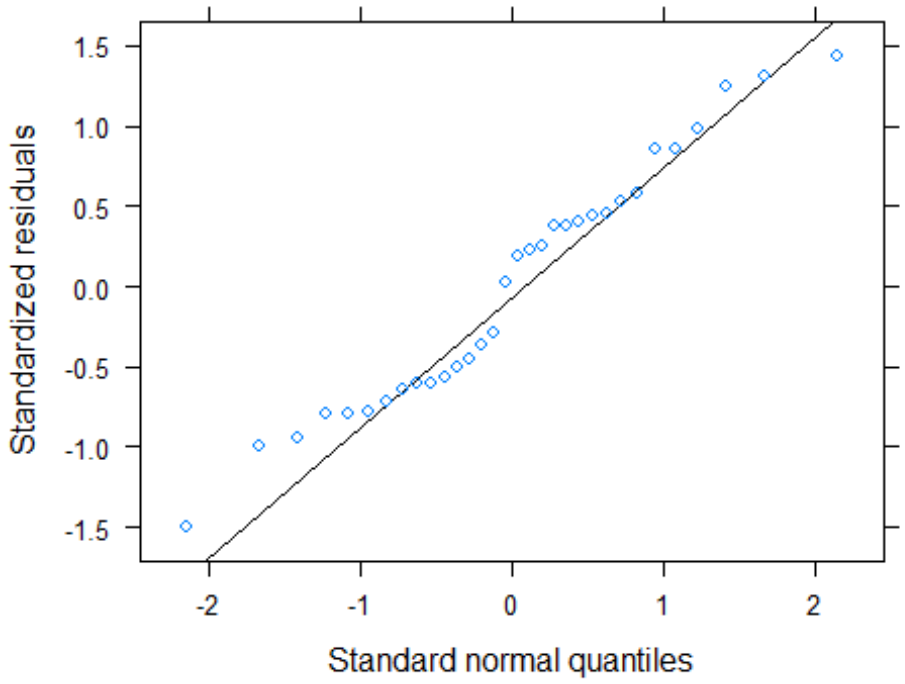
```
confint.merMod(modelP262,level=0.95,method="Wald")
```

```
##                             2.5 %      97.5 %
## .sig01                         NA          NA
## .sig02                         NA          NA
## .sigma                         NA          NA
## (Intercept)          19.4149954 19.99433498
## rescale(VAP)         -0.3263642  0.38408665
## rescale(SpermCount) -0.1352105  0.47867300
## StatusS              -0.3512049 -0.04393775
## StageB               -0.3480158 -0.10063462
```

```
plot(modelP262, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```




```
qqmath(modelP262)#Visual Check Normality assumption
```



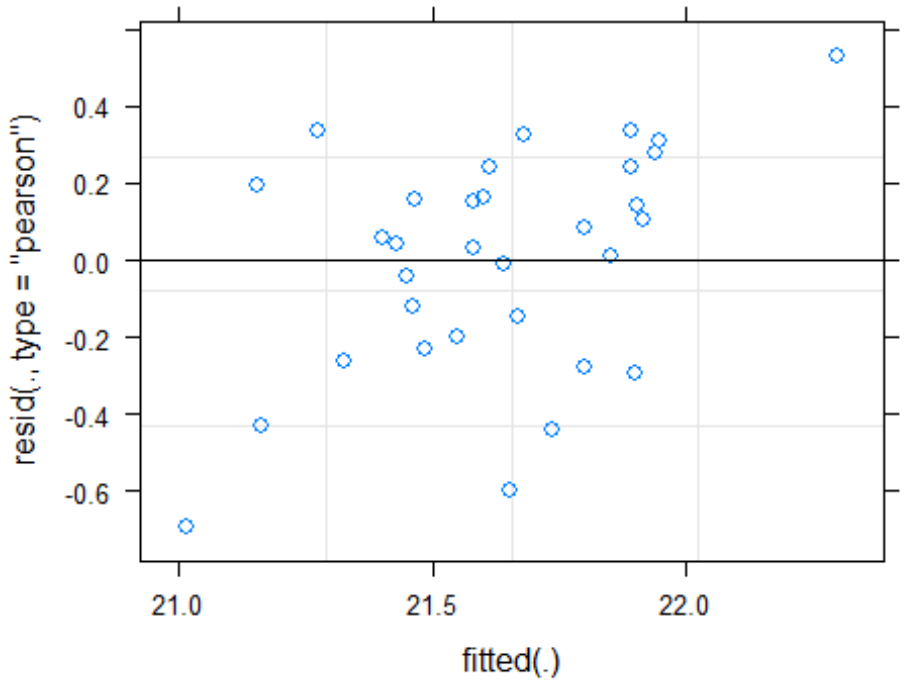

```
shapiro.test(resid(modelP262))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP262)
## W = 0.95887, p-value = 0.2558

rand(modelP262)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID  1.334      1     0.2
## Week    0.967      1     0.3

proteins[[288]]

## [1] "C1BHC3_ONCMY"

modelP288<-lmer(C1BHC3_ONCMY ~ rescale(VAP) + rescale(SpermCount) + Status +
Stage +
                  (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats
)
summary(modelP288)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: C1BHC3_ONCMY ~ rescale(VAP) + rescale(SpermCount) + Status +
```

```
##     Stage + (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 43.7
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.8638 -0.5581  0.1348  0.5493  1.4287
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.07545  0.2747
##  Week     (Intercept) 0.02381  0.1543
##  Residual             0.13888  0.3727
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error       df t value Pr(>|t|)
## (Intercept)         21.56965    0.29707 19.91700  72.608   <2e-16 ***
## rescale(VAP)         0.14878    0.37129 22.59600   0.401   0.6924
## rescale(SpermCount)  0.34502    0.33383 22.52300   1.034   0.3123
## StatusS             -0.35153    0.16706 22.78200  -2.104   0.0466 *
## StageB               0.02536    0.13701 13.92500   0.185   0.8558
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.798
## rscl(SprmC) -0.570  0.342
## StatusS     -0.067 -0.145 -0.239
## StageB      -0.100 -0.096 -0.191  0.155
```

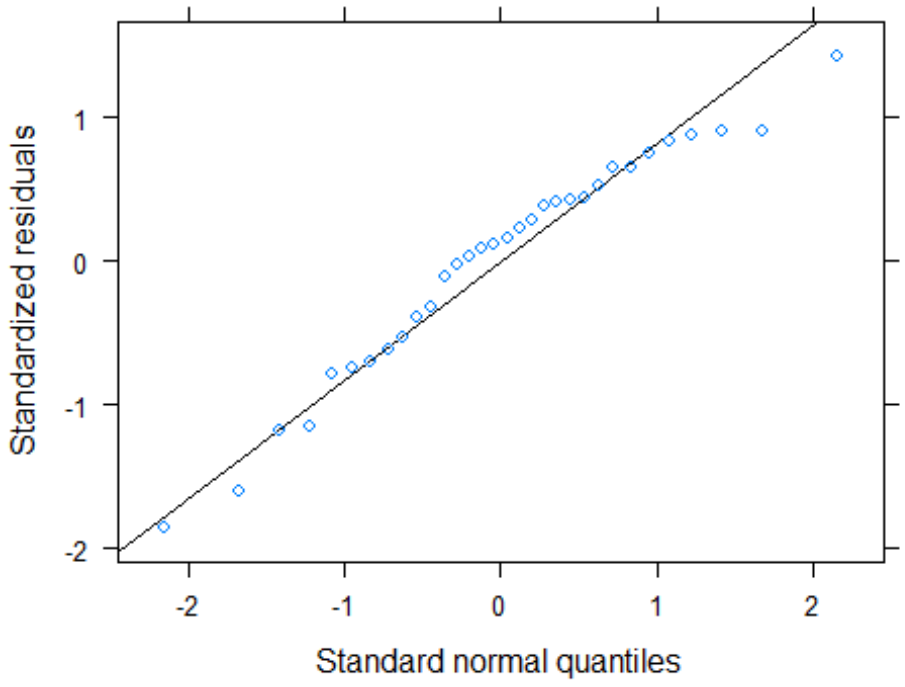
```
confint.merMod(modelP288,level=0.95,method="Wald")
```

```
##                            2.5 %      97.5 %
## .sig01                        NA          NA
## .sig02                        NA          NA
## .sigma                        NA          NA
## (Intercept)          20.9874041 22.15189784
## rescale(VAP)         -0.5789374  0.87649953
## rescale(SpermCount) -0.3092826  0.99932324
## StatusS              -0.6789607 -0.02408943
## StageB               -0.2431745  0.29389816
```

```
plot(modelP288, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```




```
qqmath(modelP288)#Visual Check Normality assumption
```




```
shapiro.test(resid(modelP288))#Test Check Normality assumption
```

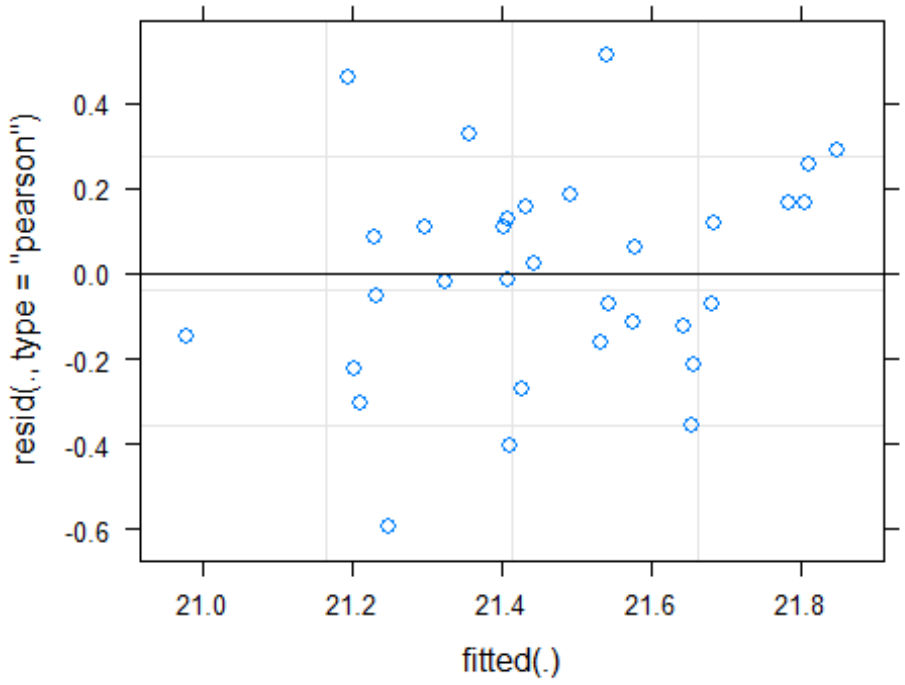
```
##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP288)
## W = 0.96199, p-value = 0.311

rand(modelP288)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID   1.55      1     0.2
## Week     0.19      1     0.7

proteins[[305]]

## [1] "F8LFR3_ONCMY"

modelP305<-lmer(F8LFR3_ONCMY ~ rescale(VAP) + rescale(SpermCount) + Status +
Stage +
                  (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats
)
summary(modelP305)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: F8LFR3_ONCMY ~ rescale(VAP) + rescale(SpermCount) + Status +
##     Stage + (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 24
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -2.04972 -0.52184  0.02098  0.54108  1.76768
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.02041  0.1428
##  Week     (Intercept) 0.00000  0.0000
##  Residual             0.08450  0.2907
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error      df t value Pr(>|t|)
## (Intercept)          21.4106     0.1948 25.7220 109.910   <2e-16 ***
## rescale(VAP)          0.1361     0.2446 25.5400   0.557   0.5827
## rescale(SpermCount)   0.5547     0.2432 24.9860   2.281   0.0313 *
## StatusS              -0.3197     0.1202 26.9990  -2.661   0.0130 *
## StageB               -0.1979     0.1061 14.3040  -1.866   0.0827 .
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
##
## Correlation of Fixed Effects:
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.793
## rscl(SprmC) -0.580  0.297
## StatusS     -0.063 -0.171 -0.240
## StageB      -0.129 -0.095 -0.191  0.137
```

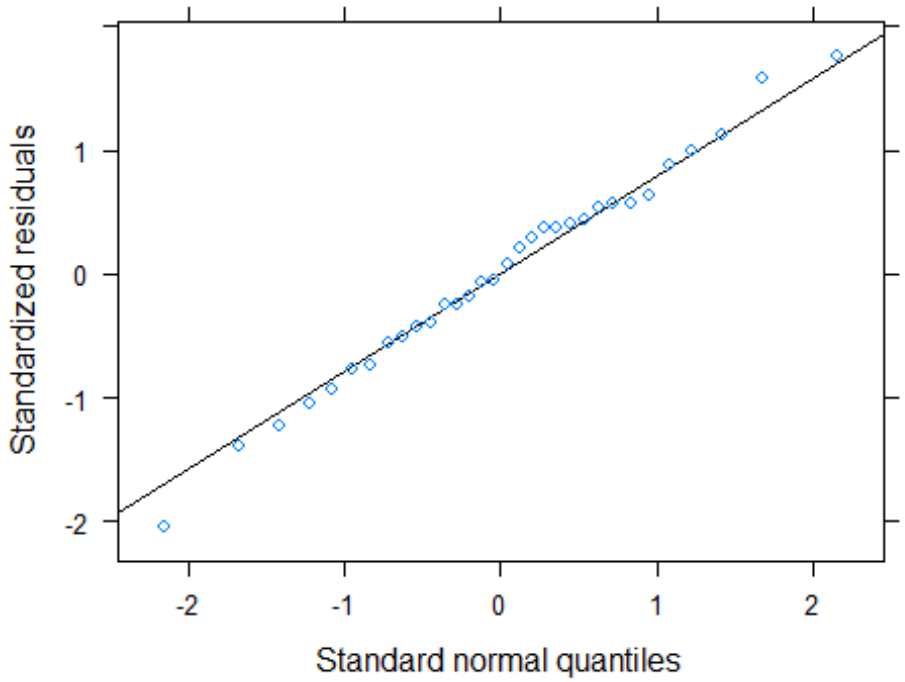
```
confint.merMod(modelP305,level=0.95,method="Wald")
```

```
##                             2.5 %       97.5 %
## .sig01                         NA           NA
## .sig02                         NA           NA
## .sigma                         NA           NA
## (Intercept)          21.02880798 21.792413542
## rescale(VAP)         -0.34330667  0.615573160
## rescale(SpermCount)   0.07809666  1.031267578
## StatusS              -0.55523854 -0.084236085
## StageB               -0.40587671  0.009993996
```

```
plot(modelP305, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```




```
qqmath(modelP305)#Visual Check Normality assumption
```



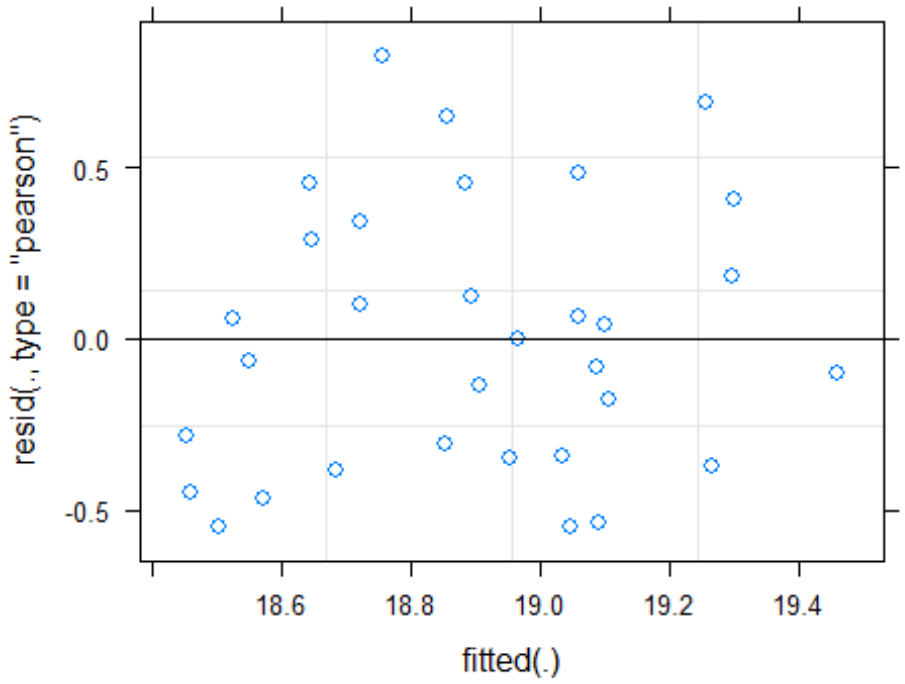

```
shapiro.test(resid(modelP305))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP305)
## W = 0.99141, p-value = 0.9953

rand(modelP305)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID  0.438      1     0.5
## Week    0.000      1     1.0

proteins[[346]]

## [1] "W5S0H9_ONCMY"

modelP346<-lmer(W5S0H9_ONCMY ~ rescale(VAP) + rescale(SpermCount) + Status +
Stage +
                  (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats
)
summary(modelP346)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: W5S0H9_ONCMY ~ rescale(VAP) + rescale(SpermCount) + Status +
```

```
##     Stage + (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 44.7
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.25600 -0.78575 -0.07219  0.68492  1.88479
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.00000  0.0000
##  Week     (Intercept) 0.05209  0.2282
##  Residual             0.19376  0.4402
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error       df t value Pr(>|t|)
## (Intercept)         19.02998    0.30524 20.52500  62.345   <2e-16 ***
## rescale(VAP)        -0.06350    0.38143 25.53600  -0.166   0.8691
## rescale(SpermCount)  0.06228    0.34842 23.62500   0.179   0.8597
## StatusS             -0.38184    0.16408 22.18300  -2.327   0.0295 *
## StageB               0.16913    0.15976 21.97800   1.059   0.3013
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.773
## rscl(SprmC) -0.552  0.309
## StatusS     -0.032 -0.177 -0.237
## StageB      -0.126 -0.088 -0.196  0.118
```

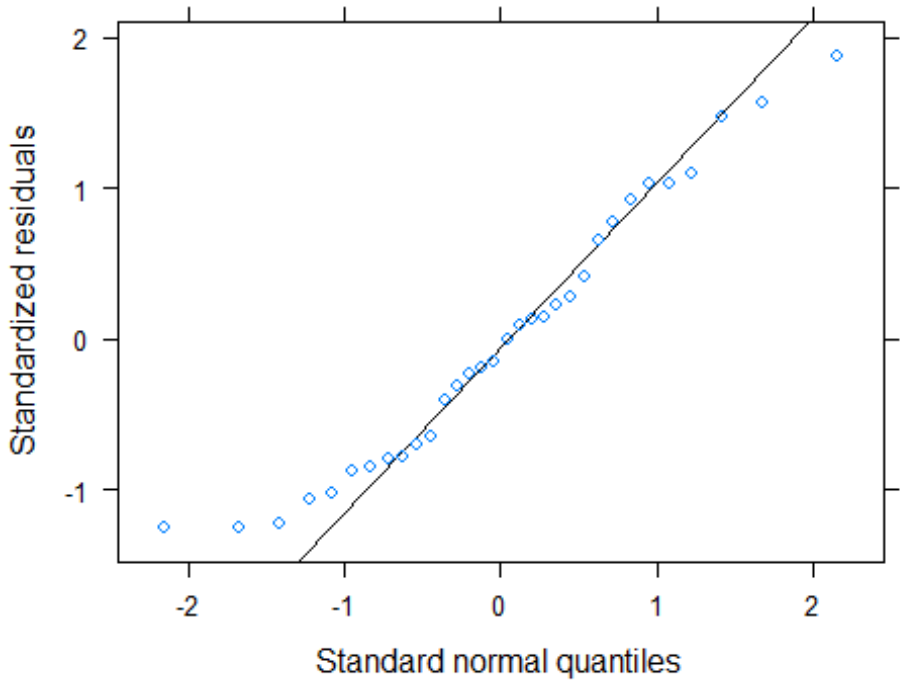
```
confint.merMod(modelP346,level=0.95,method="Wald")
```

```
##                            2.5 %      97.5 %
## .sig01                        NA          NA
## .sig02                        NA          NA
## .sigma                        NA          NA
## (Intercept)         18.4317258 19.62823949
## rescale(VAP)        -0.8110939  0.68408689
## rescale(SpermCount) -0.6206185  0.74518109
## StatusS             -0.7034202 -0.06025507
## StageB              -0.1439978  0.48226577
```

```
plot(modelP346, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```




```
qqmath(modelP346)#Visual Check Normality assumption
```




```
shapiro.test(resid(modelP346))#Test Check Normality assumption
```

```
##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP346)
## W = 0.95373, p-value = 0.1837
```

```
rand(modelP346)#Test for significance of random predictor male ID
```

```
## Analysis of Random effects Table:
##          Chi.sq Chi.DF p.value
## MaleID 2.84e-14      1     1.0
## Week   1.02e+00      1     0.3
```

TESTING FOR A CORRELATION WITH SPERM VELOCITY ACROSS MALES FROM BOTH STAGES: models with sperm velocity as significant predictor are shown below. Status and sperm number were included as fixed effects in these models, Experimental stage that ejaculates were collected is included as a cofactor

```
otherVars2<-c("VAP","SpermCount","Stage","Status" ,"(1|MaleID)","(1|Week)")
formList2<- lapply(proteins,function(x)  {
  reformulate(c(otherVars2), response=x)})
modlist2<-lapply(formList2,lmer,data=RelativeabundanceafterNormMSstats)
```

```
proteins[[36]]
```

```
## [1] "B5DGF9_SALSA"
```

```
modelP36<-lmer(B5DGF9_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status + Stage +
                 (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats)
summary(modelP36)
```

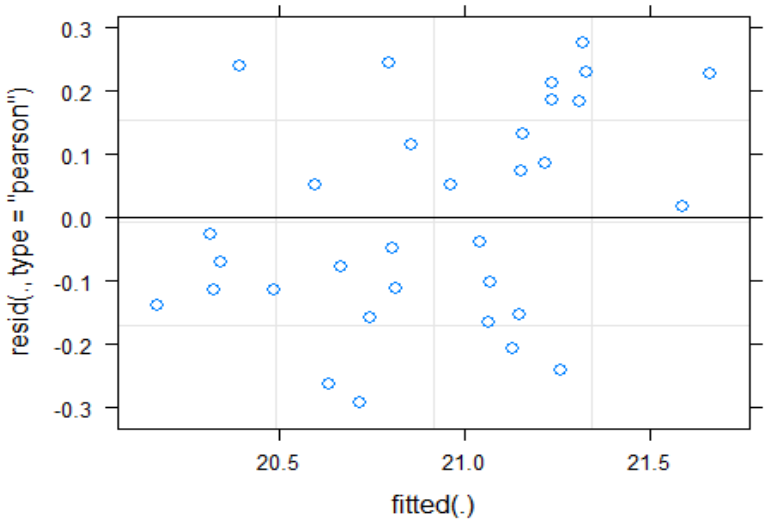
```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: B5DGF9_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
##     Stage + (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 35
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.1948 -0.4917 -0.1327  0.5874  1.1275
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.15021  0.3876
##  Week     (Intercept) 0.00000  0.0000
##  Residual             0.06032  0.2456
## Number of obs: 32, groups:  MaleID, 17; Week, 5
```

```
##
## Fixed effects:
##                     Estimate Std. Error       df t value Pr(>|t|)
## (Intercept)         20.33901    0.24312 26.51700  83.660  < 2e-16 ***
## rescale(VAP)         0.96812    0.29442 23.72600   3.288  0.00313 **
## rescale(SpermCount) -0.03988    0.24832 17.04800  -0.161  0.87429
## StatusS              0.03193    0.12980 18.08700   0.246  0.80843
## StageB               0.01344    0.09163 12.13100   0.147  0.88577
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.808
## rscl(SprmC) -0.581  0.403
## StatusS     -0.109 -0.094 -0.217
## StageB      -0.077 -0.098 -0.179  0.195
```

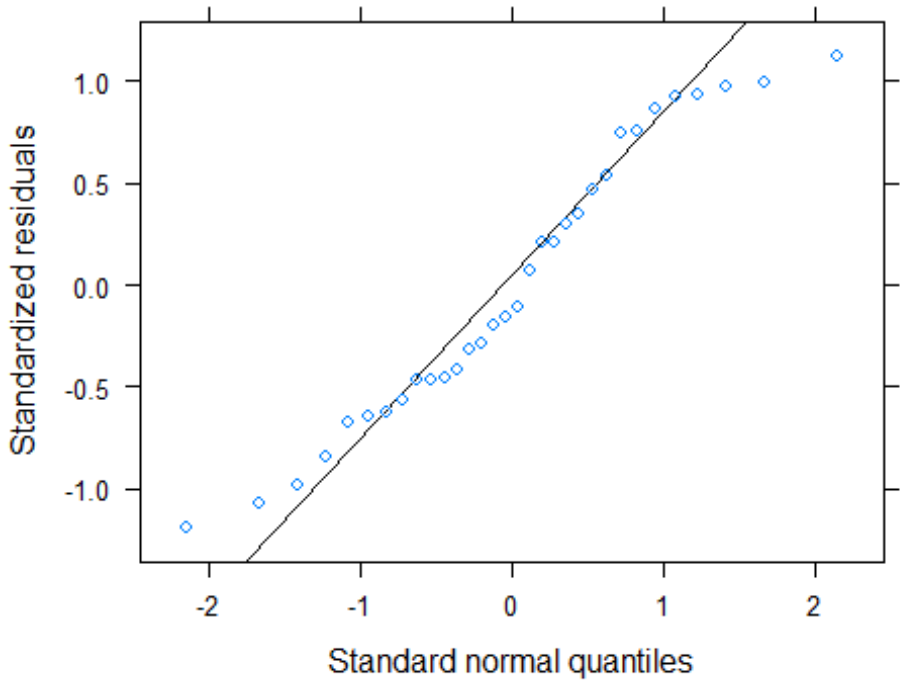
```
confint.merMod(modlist2[[36]],level=0.95,method="Wald")
```

```
##                     2.5 %       97.5 %
## .sig01                 NA           NA
## .sig02                 NA           NA
## .sigma                 NA           NA
## (Intercept) 16.921964987 20.174699097
## VAP          0.005547061  0.021917449
## SpermCount  -0.001470914  0.001248105
## StageB      -0.166150635  0.193037171
## StatusS     -0.222465752  0.286334783
```

```
plot(modelP36, results="hide", fig.show='hide')#Visual Check Variance assumption
```



```
qqmath(modelP36)#Visual Check Normality assumption
```



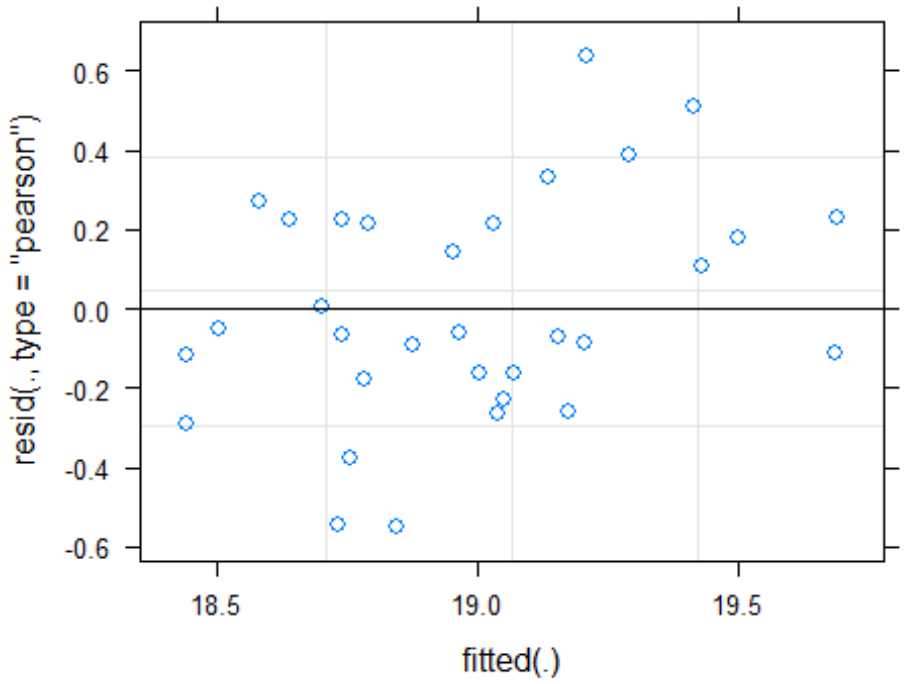

```
shapiro.test(resid(modelP36))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP36)
## W = 0.94885, p-value = 0.1337

rand(modelP36)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID    8.3      1   0.004 **
## Week      0.0      1   1.000
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

proteins[[44]]

## [1] "B5DGU8_SALSA"

modelP44<-lmer(B5DGU8_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status + S
tage +
                 (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats)
summary(modelP44)
```

```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: B5DGU8_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
##     Stage + (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 41.1
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.5482 -0.4696 -0.1828  0.6105  1.7922
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.05393  0.2322
##  Week     (Intercept) 0.04455  0.2111
##  Residual             0.12742  0.3570
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error      df t value Pr(>|t|)
## (Intercept)          18.6286     0.2905 19.3880  64.119   <2e-16 ***
## rescale(VAP)          0.9398     0.3593 25.6790   2.616   0.0147 *
## rescale(SpermCount)   0.3366     0.3157 21.0150   1.066   0.2984
## StatusS              -0.4848     0.1560 21.6010  -3.108   0.0052 **
## StageB               -0.1763     0.1310 11.7750  -1.346   0.2038
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.783
## rscl(SprmC) -0.557  0.345
## StatusS     -0.058 -0.146 -0.239
## StageB      -0.097 -0.095 -0.193  0.149
```

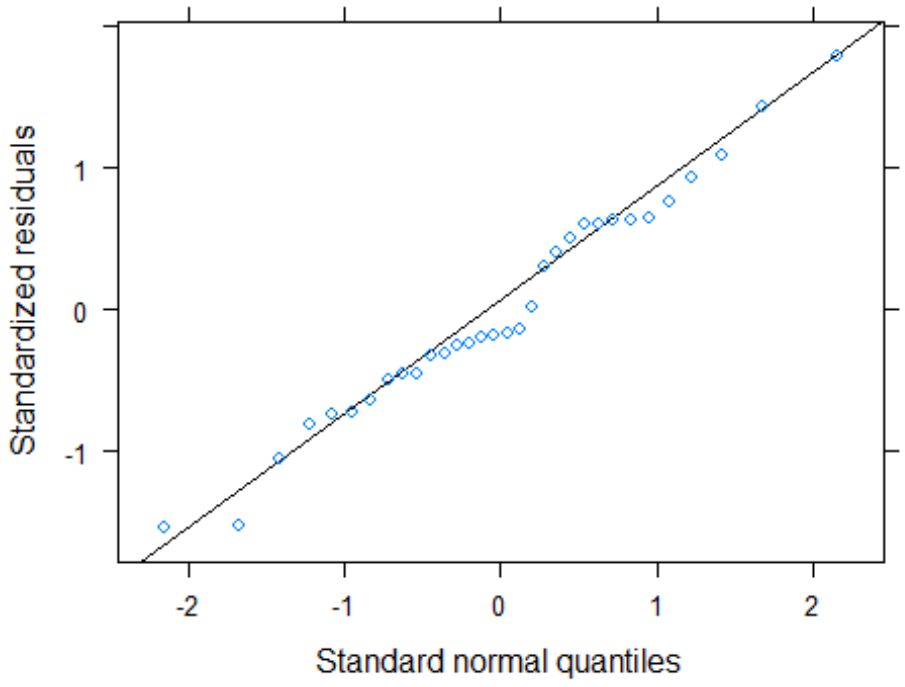
```
confint.merMod(modlist2[[44]],level=0.95,method="Wald")
```

```
##                     2.5 %       97.5 %
## .sig01                 NA           NA
## .sig02                 NA           NA
## .sigma                 NA           NA
## (Intercept) 14.7390996490 18.653610966
## VAP          0.0033425000  0.023318593
## SpermCount  -0.0007878966  0.002668326
## StageB      -0.4331273151  0.080490551
## StatusS     -0.7904589785 -0.179057485
```

```
plot(modelP44, results="hide", fig.show='hide')#Visual Check Variance assumpt
ion
```




```
qqmath(modelP44)#Visual Check Normality assumption
```




```
shapiro.test(resid(modelP44))#Test Check Normality assumption
```

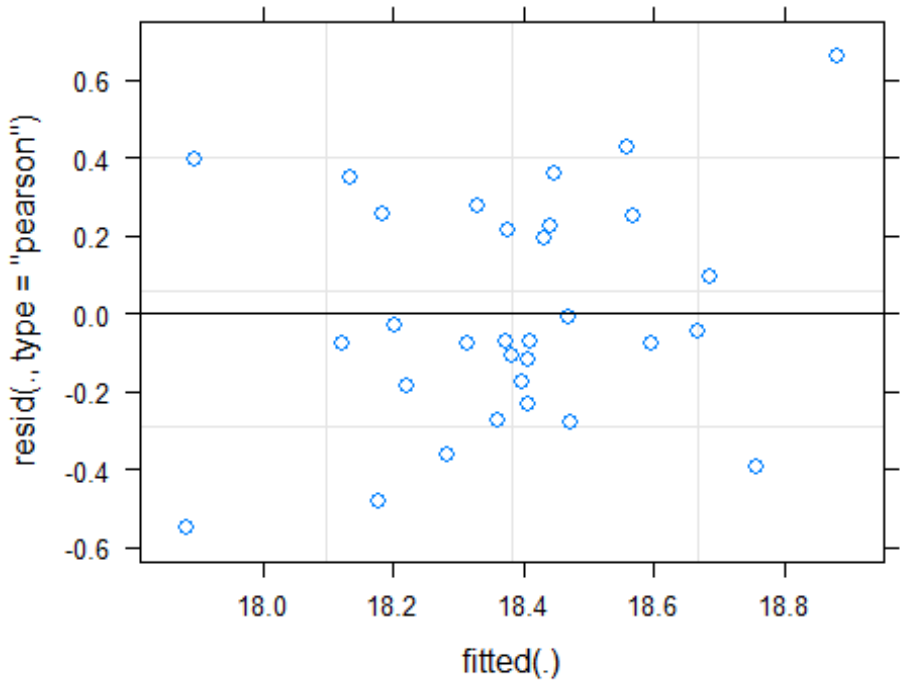
```
##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP44)
## W = 0.97742, p-value = 0.7218

rand(modelP44)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID  0.857      1     0.4
## Week    0.516      1     0.5

proteins[[48]]

## [1] "B5DH06_SALSA"

modelP48<-lmer(B5DH06_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status + S
tage + (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats)
summary(modelP48)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: B5DH06_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
##     Stage + (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 32.1
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.6622 -0.5370 -0.2159  0.6930  1.9983
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.00837  0.09149
##  Week     (Intercept) 0.04540  0.21306
##  Residual             0.11007  0.33176
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error       df t value Pr(>|t|)
## (Intercept)         18.75788    0.24773 21.77800  75.718   <2e-16 ***
## rescale(VAP)        -0.63426    0.30486 26.56100  -2.080   0.0473 *
## rescale(SpermCount) -0.18658    0.27098 24.04300  -0.689   0.4977
## StatusS             -0.05488    0.12885 22.16100  -0.426   0.6743
## StageB               0.16934    0.12078 13.18800   1.402   0.1840
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
```

```
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.764
## rscl(SprmC) -0.543  0.323
## StatusS     -0.035 -0.168 -0.239
## StageB      -0.112 -0.091 -0.196  0.126
```

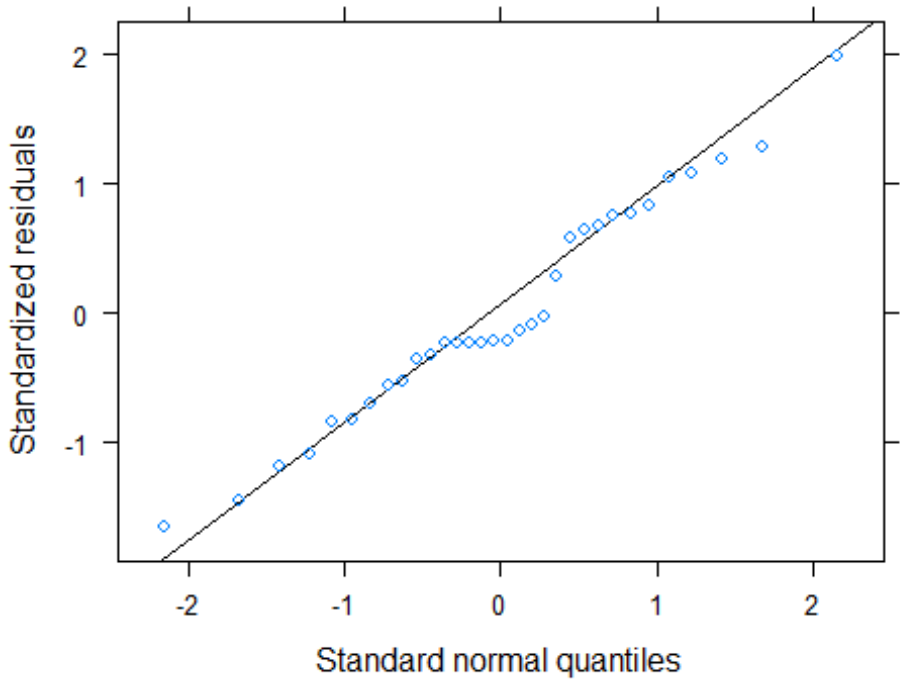
```
confint.merMod(modlist2[[48]],level=0.95,method="Wald")
```

```
##                    2.5 %        97.5 %
## .sig01                NA            NA
## .sig02                NA            NA
## .sigma                NA            NA
## (Intercept) 18.387689200 21.6942171381
## VAP         -0.017472085 -0.0005210822
## SpermCount  -0.002004758  0.0009623833
## StageB      -0.067380073  0.4060609019
## StatusS     -0.307421836  0.1976701449
```

```
plot(modelP48, results="hide", fig.show='hide')#Visual Check Variance assumpt
ion
```




```
qqmath(modelP48)#Visual Check Normality assumption
```



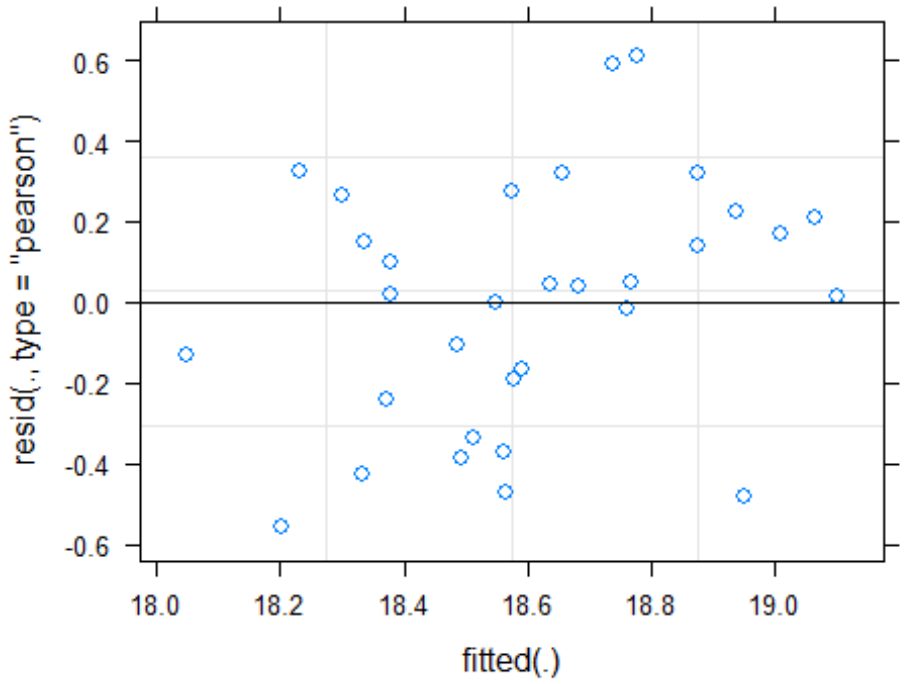

```
shapiro.test(resid(modelP48))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP48)
## W = 0.97467, p-value = 0.6367

rand(modelP48)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID 0.0487      1     0.8
## Week   2.5255      1     0.1

proteins[[70]]

## [1] "B5X1Q9_SALSA"

modelP70<-lmer(B5X1Q9_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status + S
tage + (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats)
summary(modelP70)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: B5X1Q9_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
##     Stage + (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
```

```
##
## REML criterion at convergence: 38.5
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.53129 -0.55359  0.08427  0.58108  1.67985
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.02821  0.1679
##  Week     (Intercept) 0.03494  0.1869
##  Residual             0.13294  0.3646
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error       df t value Pr(>|t|)
## (Intercept)         19.17828    0.27355 22.27200  70.110   <2e-16 ***
## rescale(VAP)        -0.72614    0.34184 25.65800  -2.124   0.0435 *
## rescale(SpermCount) -0.24693    0.30818 23.99700  -0.801   0.4308
## StatusS              0.03288    0.14968 23.02100   0.220   0.8281
## StageB              -0.17441    0.13320 13.19700  -1.309   0.2127
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.784
## rscl(SprmC) -0.559  0.326
## StatusS     -0.048 -0.161 -0.240
## StageB      -0.110 -0.093 -0.194  0.137
```

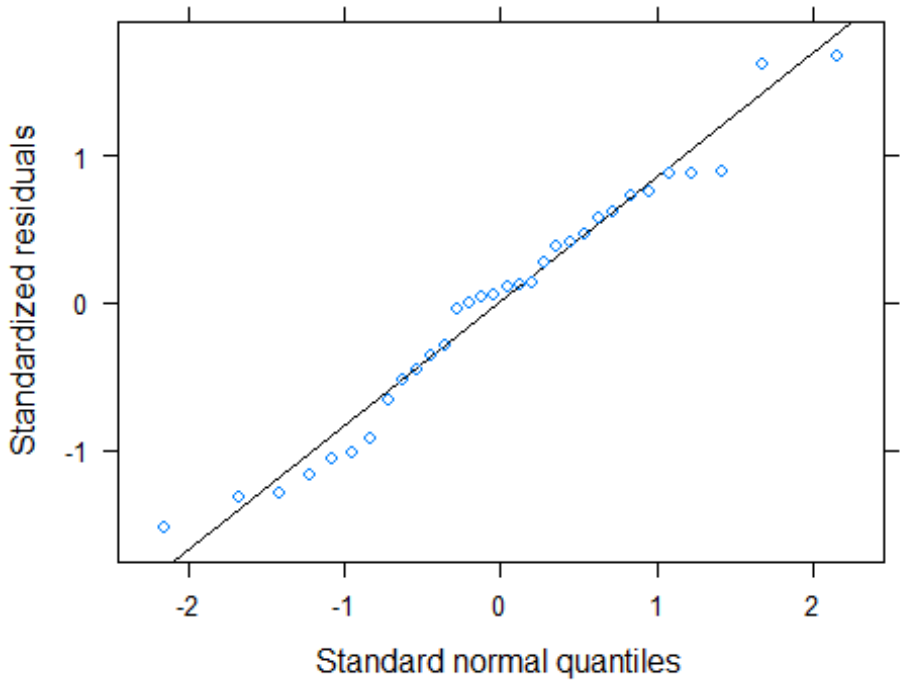
```
confint.merMod(modlist2[[70]],level=0.95,method="Wald")
```

```
##                     2.5 %        97.5 %
## .sig01                 NA            NA
## .sig02                 NA            NA
## .sigma                 NA            NA
## (Intercept) 18.806513851 22.5223561358
## VAP         -0.019803403 -0.0007962216
## SpermCount  -0.002376947  0.0009974241
## StageB      -0.435478247  0.0866527020
## StatusS     -0.260491091  0.3262548855
```

```
plot(modelP70, results="hide", fig.show='hide')#Visual Check Variance assumpt
ion
```




```
qqmath(modelP70)#Visual Check Normality assumption
```




```
shapiro.test(resid(modelP70))#Test Check Normality assumption
```

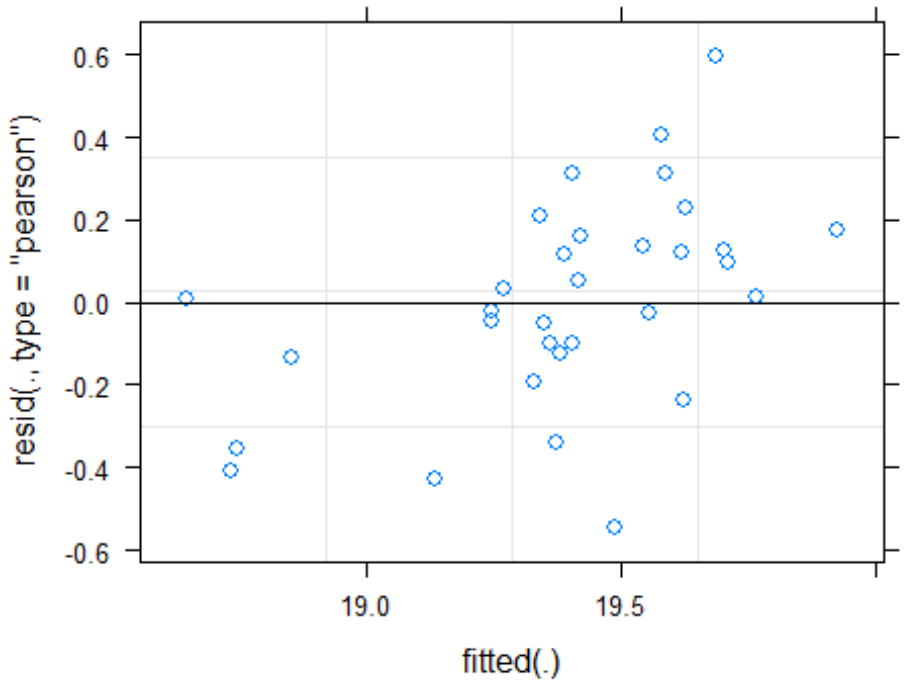
```
##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP70)
## W = 0.97037, p-value = 0.5096

rand(modelP70)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID  0.273      1     0.6
## Week    0.930      1     0.3

proteins[[72]]

## [1] "B5X1V0_SALSA"

modelP72<-lmer(B5X1V0_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status + S
tage + (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats)
summary(modelP72)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: B5X1V0_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
##     Stage + (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 44.1
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.59777 -0.37443  0.02837  0.41985  1.74710
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.1324   0.3639
##  Week     (Intercept) 0.0000   0.0000
##  Residual             0.1177   0.3430
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error       df t value Pr(>|t|)
## (Intercept)         19.89964    0.28968 26.95900  68.696   <2e-16 ***
## rescale(VAP)        -0.77595    0.36007 26.72900  -2.155   0.0403 *
## rescale(SpermCount) -0.33367    0.32416 18.77800  -1.029   0.3164
## StatusS              0.11415    0.16691 21.74400   0.684   0.5013
## StageB               0.05341    0.12695 11.05100   0.421   0.6820
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
```

```
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.811
## rscl(SprmC) -0.582  0.358
## StatusS     -0.090 -0.127 -0.233
## StageB      -0.092 -0.100 -0.186  0.174
```

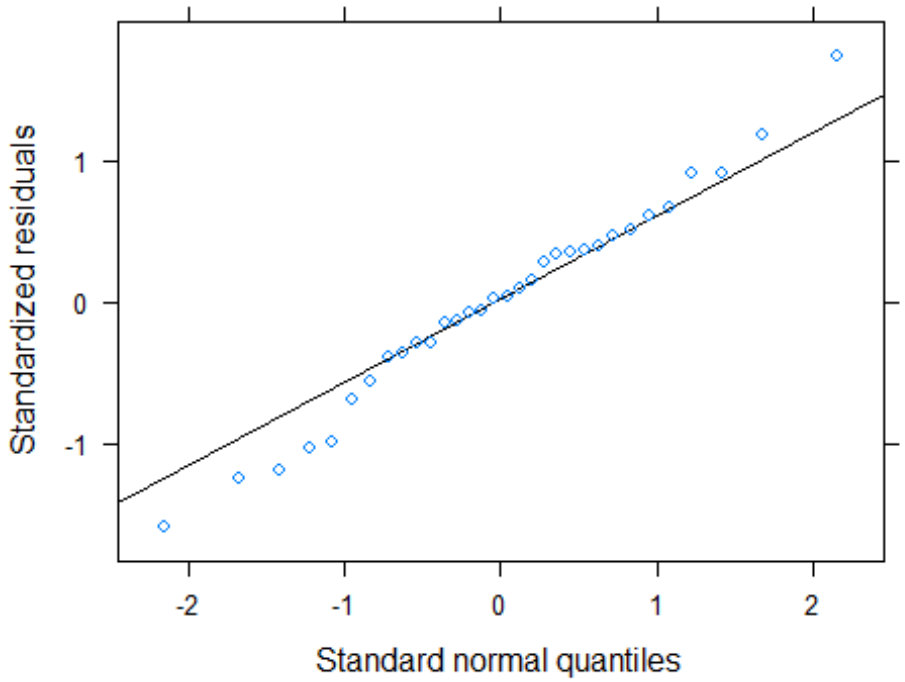
```
confint.merMod(modlist2[[72]],level=0.95,method="Wald")
```

```
##                    2.5 %        97.5 %
## .sig01                NA            NA
## .sig02                NA            NA
## .sigma                NA            NA
## (Intercept) 19.544723533 23.5028776379
## VAP         -0.021016507 -0.0009961732
## SpermCount  -0.002706747  0.0008426581
## StageB      -0.195404296  0.3022214507
## StatusS     -0.212989668  0.4412879546
```

```
plot(modelP72, results="hide", fig.show='hide')#Visual Check Variance assumpt
ion
```




```
qqmath(modelP72)#Visual Check Normality assumption
```



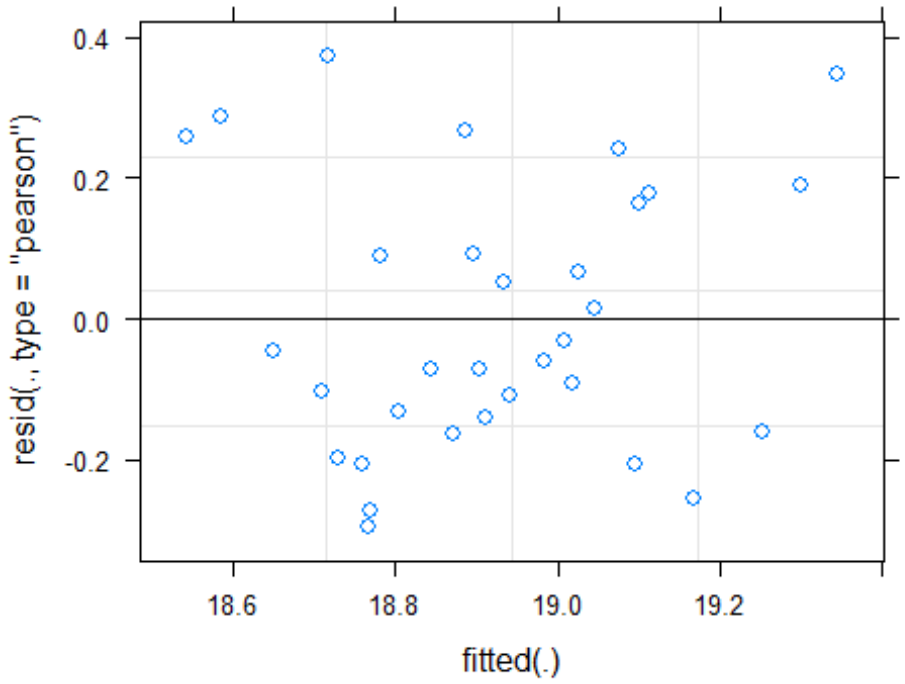

```
shapiro.test(resid(modelP72))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP72)
## W = 0.98624, p-value = 0.947

rand(modelP72)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID   3.06      1    0.08 .
## Week     0.00      1    1.00
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

proteins[[73]]

## [1] "B5X1X1_SALSA"

modelP73<-lmer(B5X1X1_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status + S
tage + (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats)
summary(modelP73)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: B5X1X1_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
```

```
##     Stage + (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 4.9
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.3864 -0.6774 -0.2505  0.7819  1.7510
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.000000 0.0000
##  Week     (Intercept) 0.008083 0.0899
##  Residual             0.045880 0.2142
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error       df t value Pr(>|t|)
## (Intercept)         19.39420    0.14323 21.49600 135.407  < 2e-16 ***
## rescale(VAP)        -0.38471    0.18049 23.87100  -2.131  0.04355 *
## rescale(SpermCount) -0.23639    0.16883 24.70600  -1.400  0.17390
## StatusS             -0.25942    0.07977 23.31600  -3.252  0.00347 **
## StageB              -0.09142    0.07771 23.10700  -1.176  0.25139
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.780
## rscl(SprmC) -0.561  0.302
## StatusS     -0.035 -0.180 -0.237
## StageB      -0.132 -0.088 -0.195  0.118
```

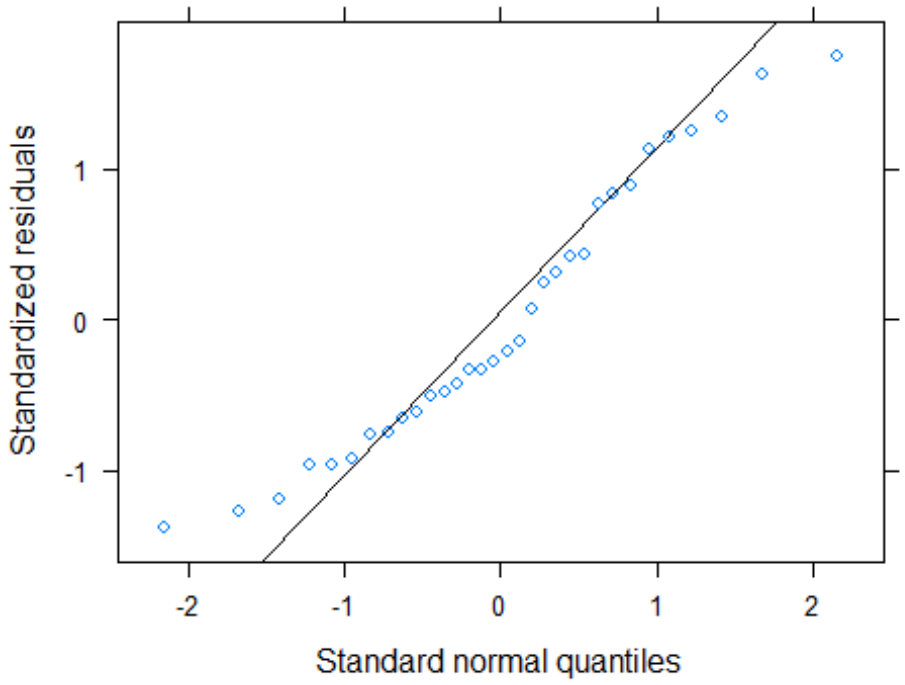
```
confint.merMod(modlist2[[73]],level=0.95,method="Wald")
```

```
##                    2.5 %        97.5 %
## .sig01                NA            NA
## .sig02                NA            NA
## .sigma                NA            NA
## (Intercept) 19.257306575 21.2149231488
## VAP         -0.010474632 -0.0004390370
## SpermCount  -0.001584633  0.0002640119
## StageB      -0.243733477  0.0608874526
## StatusS     -0.415765638 -0.1030680565
```

```
plot(modelP73, results="hide", fig.show='hide')#Visual Check Variance assumpt
ion
```




```
qqmath(modelP73)#Visual Check Normality assumption
```




```
shapiro.test(resid(modelP73))#Test Check Normality assumption
```

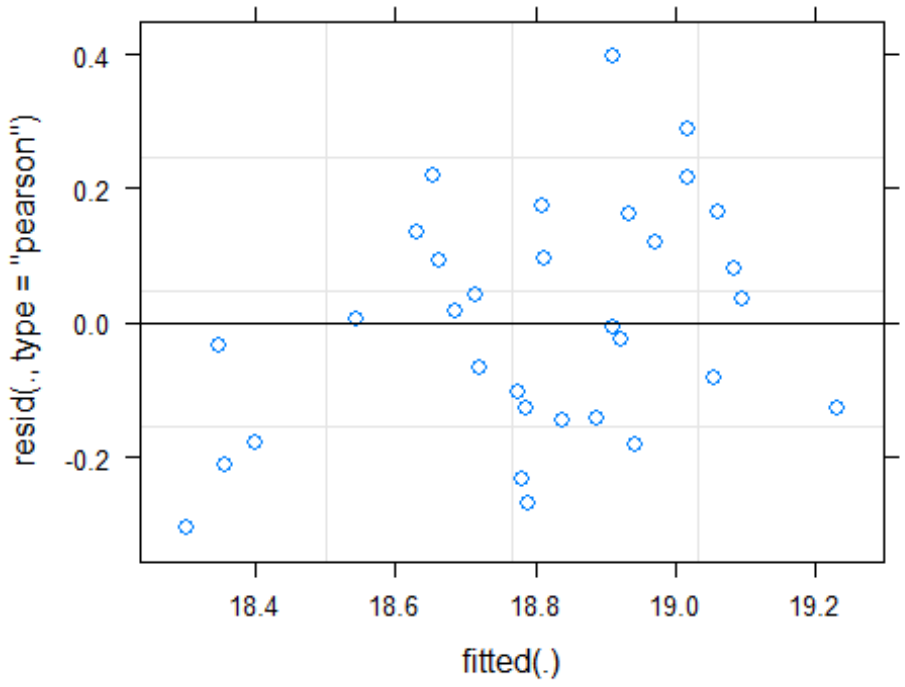
```
##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP73)
## W = 0.94871, p-value = 0.1324

rand(modelP73)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##          Chi.sq Chi.DF p.value
## MaleID 7.11e-15      1     1.0
## Week   7.93e-01      1     0.4

proteins[[75]]

## [1] "B5X205_SALSA"

modelP75<-lmer(B5X205_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status + S
tage + (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats)
summary(modelP75)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: B5X205_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
##     Stage + (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 18.1
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.37822 -0.59098 -0.00323  0.55747  1.79125
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.02828  0.1682
##  Week     (Intercept) 0.02448  0.1565
##  Residual             0.04958  0.2227
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error        df t value Pr(>|t|)
## (Intercept)         19.141225   0.192784 21.621000  99.288   <2e-16 ***
## rescale(VAP)        -0.644951   0.234853 26.265000  -2.746   0.0107 *
## rescale(SpermCount)  0.097099   0.201903 21.702000   0.481   0.6354
## StatusS              0.008176   0.100494 23.366000   0.081   0.9359
## StageB              -0.021085   0.081955 12.711000  -0.257   0.8011
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
```

```
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.776
## rscl(SprmC) -0.551  0.359
## StatusS     -0.063 -0.136 -0.237
## StageB      -0.089 -0.096 -0.192  0.157
```

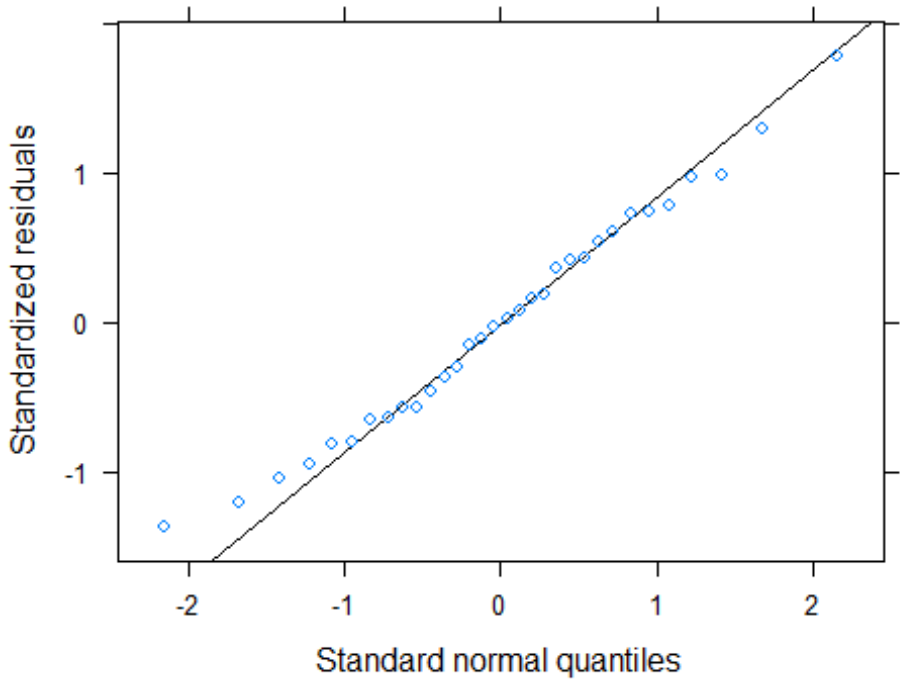
```
confint.merMod(modlist2[[75]],level=0.95,method="Wald")
```

```
##                     2.5 %       97.5 %
## .sig01                 NA           NA
## .sig02                 NA           NA
## .sigma                 NA           NA
## (Intercept) 19.0160971011 21.579303834
## VAP         -0.0156773708 -0.002619105
## SpermCount  -0.0008341441  0.001376595
## StageB      -0.1817130840  0.139542938
## StatusS     -0.1887888615  0.205140593
```

```
plot(modelP75, results="hide", fig.show='hide')#Visual Check Variance assumption
```




```
qqmath(modelP75)#Visual Check Normality assumption
```



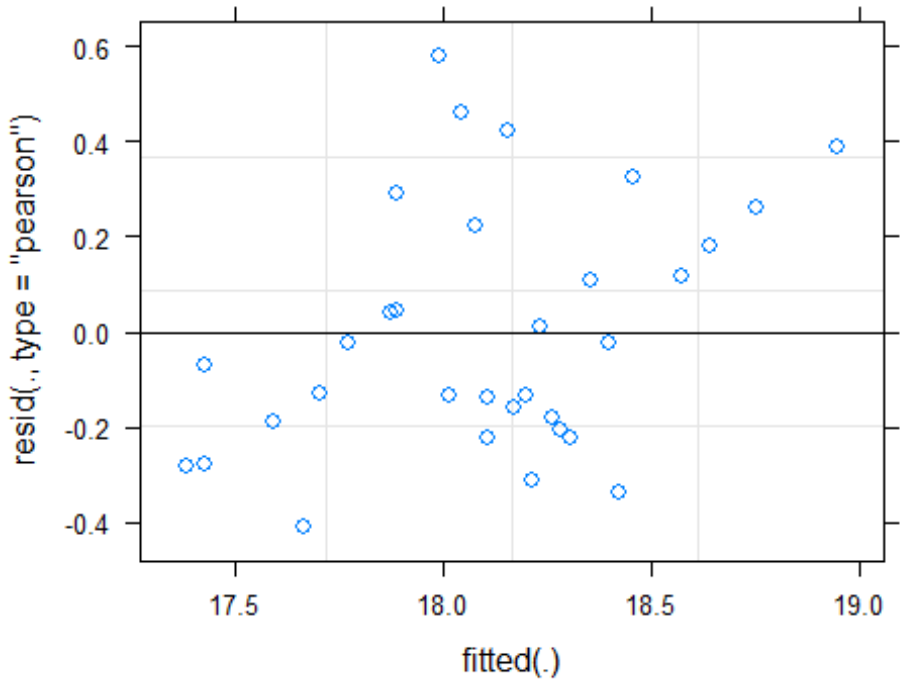

```
shapiro.test(resid(modelP75))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP75)
## W = 0.98492, p-value = 0.9232

rand(modelP75)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID   1.38      1     0.2
## Week     1.31      1     0.3

proteins[[79]]

## [1] "B5X2I6_SALSA"

modelP79<-lmer(B5X2I6_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status + S
tage + (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats)
summary(modelP79)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: B5X2I6_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
##     Stage + (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
```

```
##
## REML criterion at convergence: 43.5
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.1888 -0.5610 -0.1355  0.5518  1.6799
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.09837  0.3136
##  Week     (Intercept) 0.03337  0.1827
##  Residual             0.12020  0.3467
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error      df t value Pr(>|t|)
## (Intercept)          17.5962     0.3001 21.2820  58.640   <2e-16 ***
## rescale(VAP)          0.8289     0.3703 24.6390   2.239   0.0345 *
## rescale(SpermCount)   0.5706     0.3221 21.5970   1.771   0.0906 .
## StatusS              -0.2806     0.1629 22.8790  -1.722   0.0985 .
## StageB               -0.1601     0.1280 14.0060  -1.251   0.2315
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.794
## rscl(SprmC) -0.566  0.363
## StatusS     -0.075 -0.130 -0.235
## StageB      -0.089 -0.097 -0.189  0.166
```

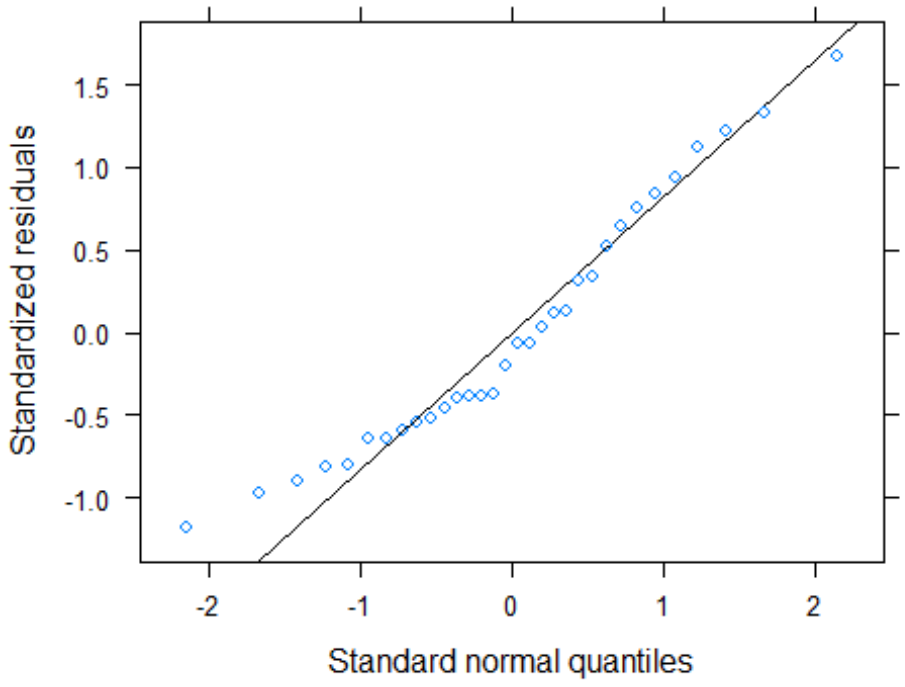
```
confint.merMod(modlist2[[79]],level=0.95,method="Wald")
```

```
##                     2.5 %       97.5 %
## .sig01                 NA           NA
## .sig02                 NA           NA
## .sigma                 NA           NA
## (Intercept) 13.7250069752 17.776169968
## VAP          0.0014636942  0.022050866
## SpermCount  -0.0001698322  0.003357356
## StageB      -0.4109561797  0.090752238
## StatusS     -0.5997920975  0.038687559
```

```
plot(modelP79, results="hide", fig.show='hide')#Visual Check Variance assumpt
ion
```




```
qqmath(modelP79)#Visual Check Normality assumption
```




```
shapiro.test(resid(modelP79))#Test Check Normality assumption
```

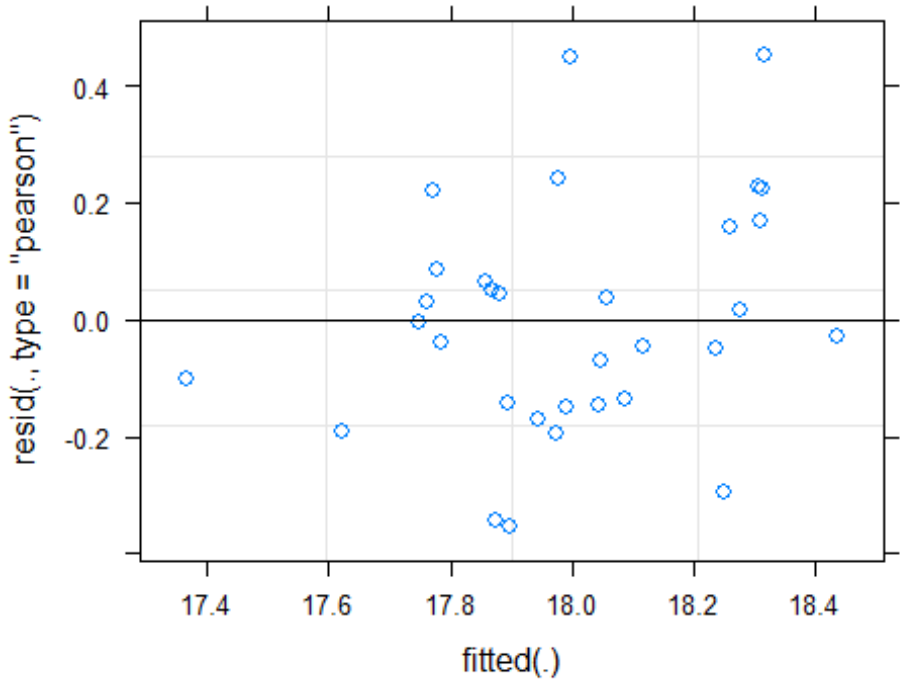
```
##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP79)
## W = 0.95071, p-value = 0.1509

rand(modelP79)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID  2.929      1    0.09 .
## Week    0.387      1    0.53
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

proteins[[81]]

## [1] "B5X2Q5_SALSA"

modelP81<-lmer(B5X2Q5_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status + S
tage + (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats)
summary(modelP81)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: B5X2Q5_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
##     Stage + (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 20.7
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.4414 -0.5852 -0.0651  0.4188  1.8405
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.01669  0.1292
##  Week     (Intercept) 0.04217  0.2054
##  Residual             0.06079  0.2466
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error       df t value Pr(>|t|)
## (Intercept)         18.65253    0.20762 20.35900  89.842  < 2e-16 ***
## rescale(VAP)        -0.83373    0.24814 26.95300  -3.360  0.00234 **
## rescale(SpermCount) -0.58598    0.21317 23.00700  -2.749  0.01143 *
## StatusS             -0.04942    0.10351 22.78300  -0.477  0.63763
## StageB               0.13665    0.09028 14.12800   1.514  0.15216
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
##
## Correlation of Fixed Effects:
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.750
## rscl(SprmC) -0.530  0.346
## StatusS     -0.045 -0.150 -0.240
## StageB      -0.093 -0.094 -0.195  0.141
```

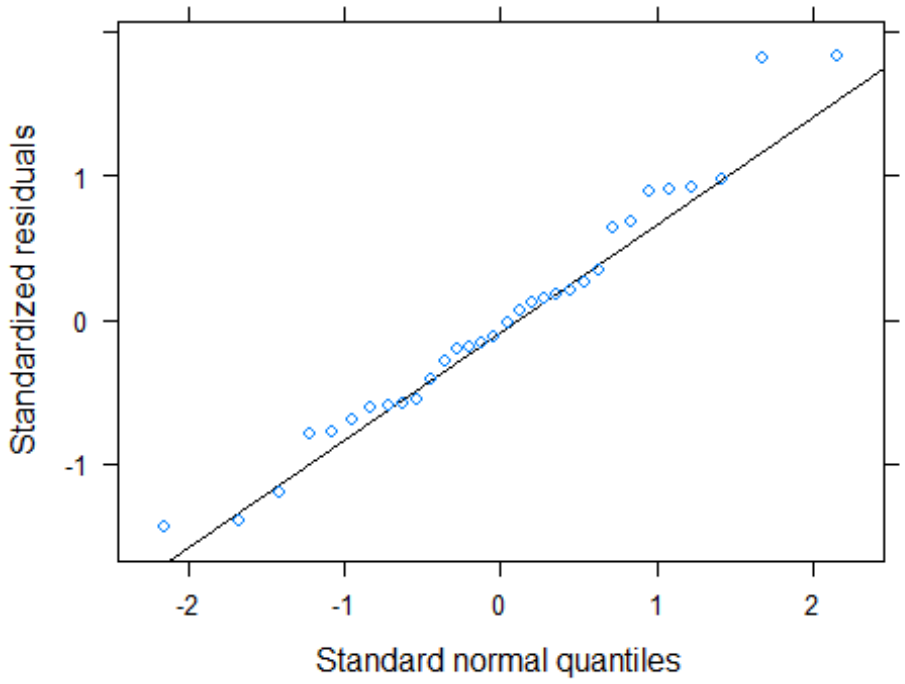
```
confint.merMod(modlist2[[81]],level=0.95,method="Wald")
```

```
##                     2.5 %        97.5 %
## .sig01                 NA            NA
## .sig02                 NA            NA
## .sigma                 NA            NA
## (Intercept) 19.164392685 21.8659860586
## VAP         -0.018724545 -0.0049274110
## SpermCount  -0.002803879 -0.0004697342
## StageB      -0.040291499  0.3135849369
## StatusS     -0.252293991  0.1534623380
```

```
plot(modelP81, results="hide", fig.show='hide')#Visual Check Variance assumpt
ion
```




```
qqmath(modelP81)#Visual Check Normality assumption
```



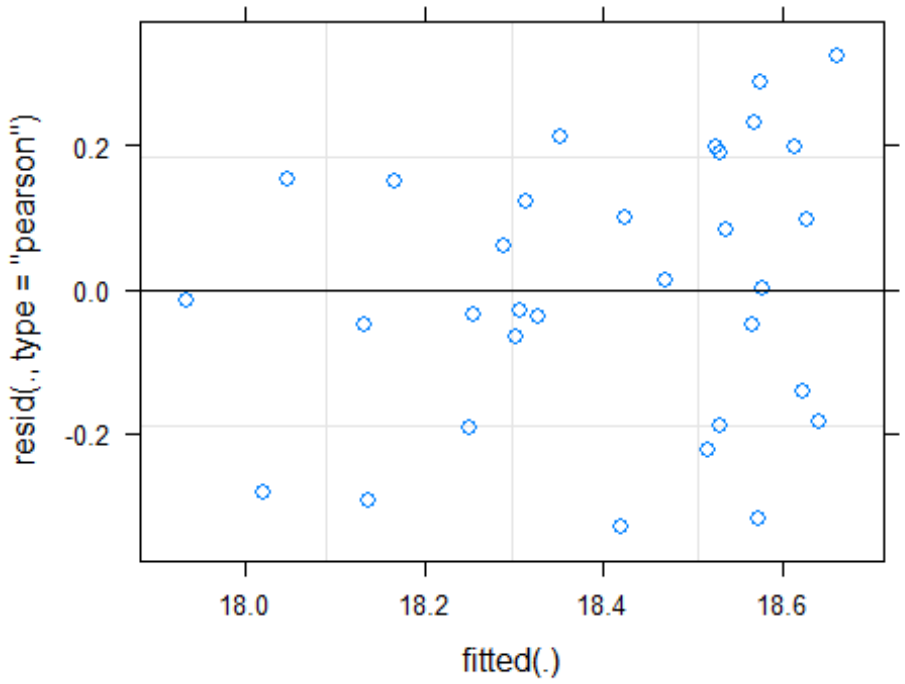

```
shapiro.test(resid(modelP81))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP81)
## W = 0.96898, p-value = 0.4719

rand(modelP81)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID  0.558      1     0.5
## Week    2.638      1     0.1

proteins[[97]]

## [1] "B5X499_SALSA"

modelP97<-lmer(B5X499_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status + S
tage + (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats)
summary(modelP97)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: B5X499_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
##     Stage + (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
```

```
##
## REML criterion at convergence: 11.5
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.50904 -0.68880 -0.02722  0.69079  1.48553
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.005872 0.07663
##  Week     (Intercept) 0.028363 0.16841
##  Residual             0.047998 0.21908
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                      Estimate Std. Error        df t value Pr(>|t|)
## (Intercept)         18.657569   0.173200 21.462000 107.723   <2e-16 ***
## rescale(VAP)        -0.451596   0.208832 26.734000  -2.162   0.0397 *
## rescale(SpermCount) -0.201396   0.182084 23.838000  -1.106   0.2797
## StatusS             -0.002506   0.086951 21.461000  -0.029   0.9773
## StageB               0.185101   0.079893 11.307000   2.317   0.0402 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.750
## rscl(SprmC) -0.531  0.332
## StatusS     -0.036 -0.162 -0.239
## StageB      -0.103 -0.092 -0.197  0.130
```

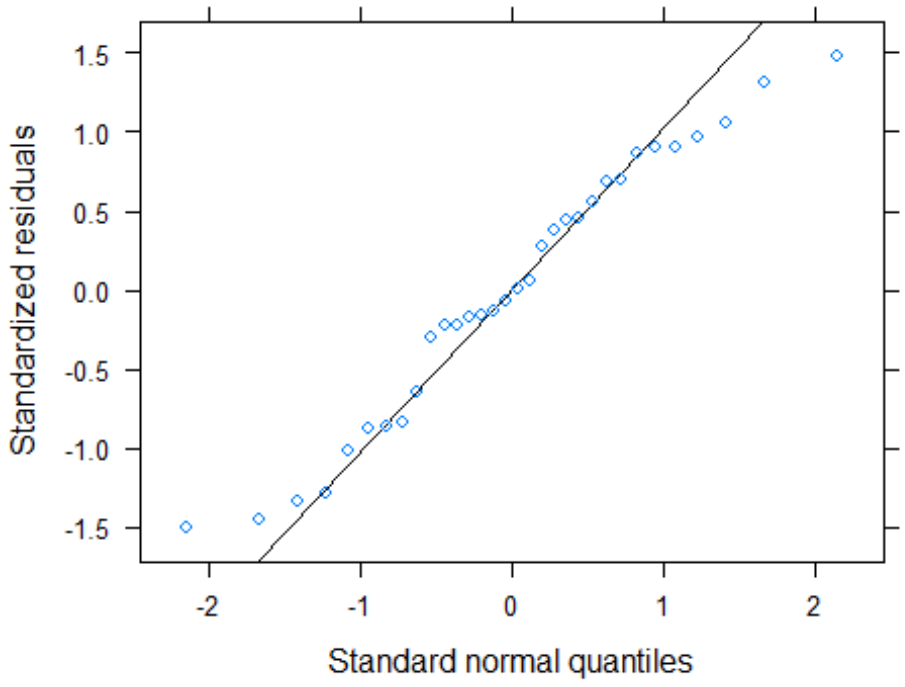
```
confint.merMod(modlist2[[97]],level=0.95,method="Wald")
```

```
##                     2.5 %        97.5 %
## .sig01                 NA            NA
## .sig02                 NA            NA
## .sigma                 NA            NA
## (Intercept) 18.472479898 20.7406056451
## VAP         -0.012211320 -0.0005999012
## SpermCount  -0.001559425  0.0004343054
## StageB       0.028512747  0.3416894511
## StatusS     -0.172926964  0.1679143482
```

```
plot(modelP97, results="hide", fig.show='hide')#Visual Check Variance assumpt
ion
```




```
qqmath(modelP97)#Visual Check Normality assumption
```




```
shapiro.test(resid(modelP97))#Test Check Normality assumption
```

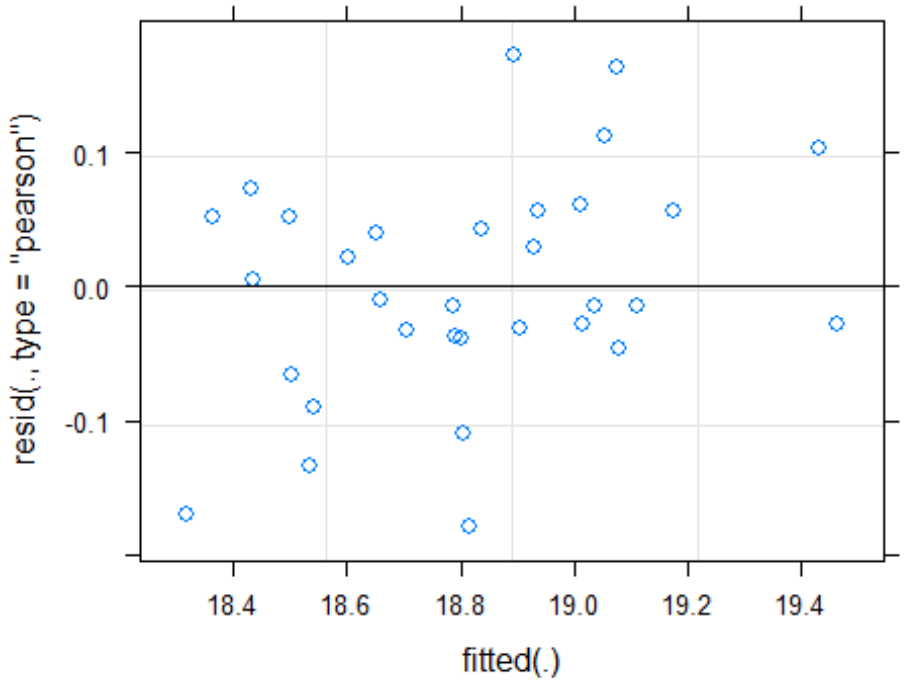
```
##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP97)
## W = 0.96321, p-value = 0.3354

rand(modelP97)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID 0.0746      1    0.78
## Week   3.0087      1    0.08 .
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

proteins[[117]]

## [1] "B5X6Z9_SALSA"

modelP117<-lmer(B5X6Z9_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
Stage + (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats)
summary(modelP117)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: B5X6Z9_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
##     Stage + (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 10.7
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.38177 -0.28707 -0.09237  0.41034  1.33324
##
## Random effects:
##  Groups   Name        Variance  Std.Dev.
##  MaleID   (Intercept) 9.002e-02 3.000e-01
##  Week     (Intercept) 6.739e-16 2.596e-08
##  Residual             1.685e-02 1.298e-01
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error       df t value Pr(>|t|)
## (Intercept)         18.53220    0.15012 25.44600 123.449   <2e-16 ***
## rescale(VAP)         0.42573    0.17276 19.08600   2.464   0.0234 *
## rescale(SpermCount) -0.03433    0.13819 14.87200  -0.248   0.8072
## StatusS              0.09164    0.07261 15.02400   1.262   0.2262
## StageB               0.03489    0.04872 11.93700   0.716   0.4877
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
##
## Correlation of Fixed Effects:
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.781
## rscl(SprmC) -0.567  0.442
## StatusS     -0.123 -0.062 -0.198
## StageB      -0.066 -0.095 -0.172  0.210
```

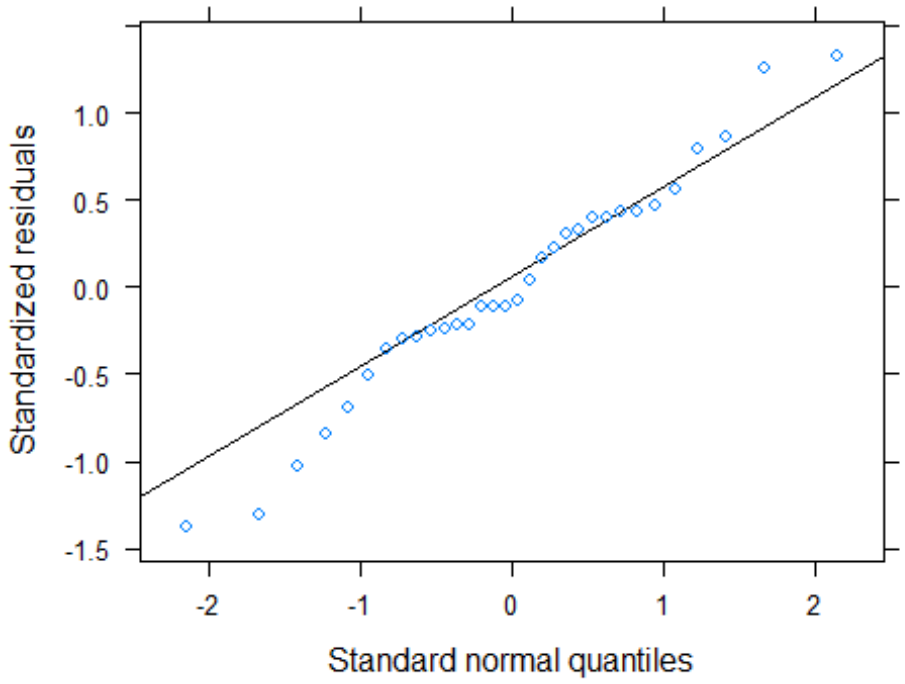
```
confint.merMod(modlist2[[117]],level=0.95,method="Wald")
```

```
##                      2.5 %       97.5 %
## .sig01                  NA           NA
## .sig02                  NA           NA
## .sigma                  NA           NA
## (Intercept) 16.7922544749 1.871461e+01
## VAP          0.0012358365 1.084155e-02
## SpermCount  -0.0008524324 6.606358e-04
## StageB      -0.0606025058 1.303810e-01
## StatusS     -0.0506717714 2.339534e-01
```

```
plot(modelP117, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```




```
qqmath(modelP117)#Visual Check Normality assumption
```



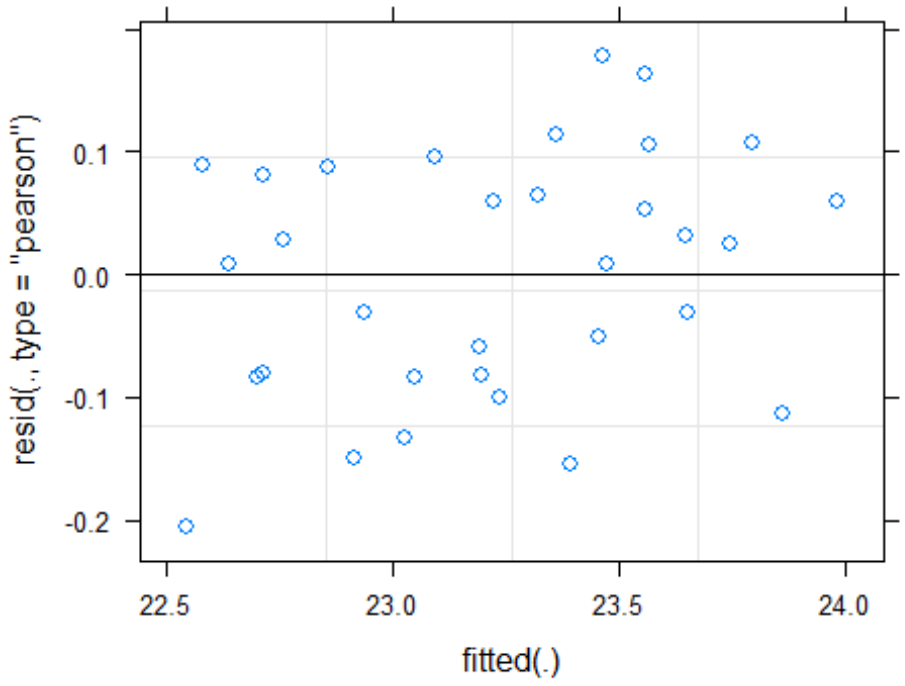

```
shapiro.test(resid(modelP117))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP117)
## W = 0.97646, p-value = 0.692

rand(modelP117)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##          Chi.sq Chi.DF p.value
## MaleID 1.55e+01      1   8e-05 ***
## Week   4.26e-14      1       1
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

proteins[[126]]

## [1] "B5X8G7_SALSA"

modelP126<-lmer(B5X8G7_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
Stage + (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats)
summary(modelP126)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: B5X8G7_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
```

```
##     Stage + (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 23.1
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.3264 -0.5267  0.1091  0.5326  1.1477
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.15504  0.3937
##  Week     (Intercept) 0.00000  0.0000
##  Residual             0.02422  0.1556
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error       df t value Pr(>|t|)
## (Intercept)         23.52610    0.18681 25.12700 125.938   <2e-16 ***
## rescale(VAP)        -0.46926    0.21127 17.23000  -2.221    0.040 *
## rescale(SpermCount)  0.03880    0.16729 13.50800   0.232    0.820
## StatusS             -0.05253    0.08793 13.56300  -0.597    0.560
## StageB              -0.04675    0.05848 10.96900  -0.799    0.441
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.770
## rscl(SprmC) -0.560  0.450
## StatusS     -0.125 -0.056 -0.193
## StageB      -0.064 -0.094 -0.170  0.213
```

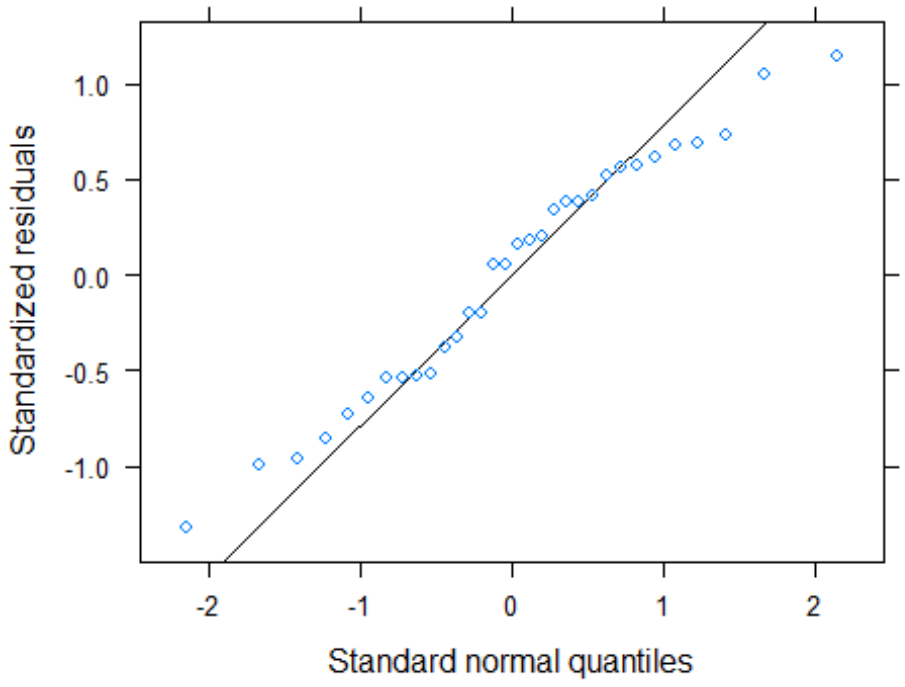
```
confint.merMod(modlist2[[126]],level=0.95,method="Wald")
```

```
##                     2.5 %        97.5 %
## .sig01                 NA            NA
## .sig02                 NA            NA
## .sigma                 NA            NA
## (Intercept) 23.2060165290 25.5619928974
## VAP         -0.0125296391 -0.0007827232
## SpermCount  -0.0008075167  0.0010242691
## StageB      -0.1613664773  0.0678735351
## StatusS     -0.2248715821  0.1198054996
```

```
plot(modelP126, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```




```
qqmath(modelP126)#Visual Check Normality assumption
```




```
shapiro.test(resid(modelP126))#Test Check Normality assumption
```

```
##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP126)
## W = 0.96847, p-value = 0.4583
```

```
rand(modelP126)#Test for significance of random predictor male ID
```

```
## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID   13.9      1   2e-04 ***
## Week      0.0      1       1
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
proteins[[137]]
```

```
## [1] "B5X9M8_SALSA"
```

```
modelP137<-lmer(B5X9M8_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
Stage + (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats)
summary(modelP137)
```

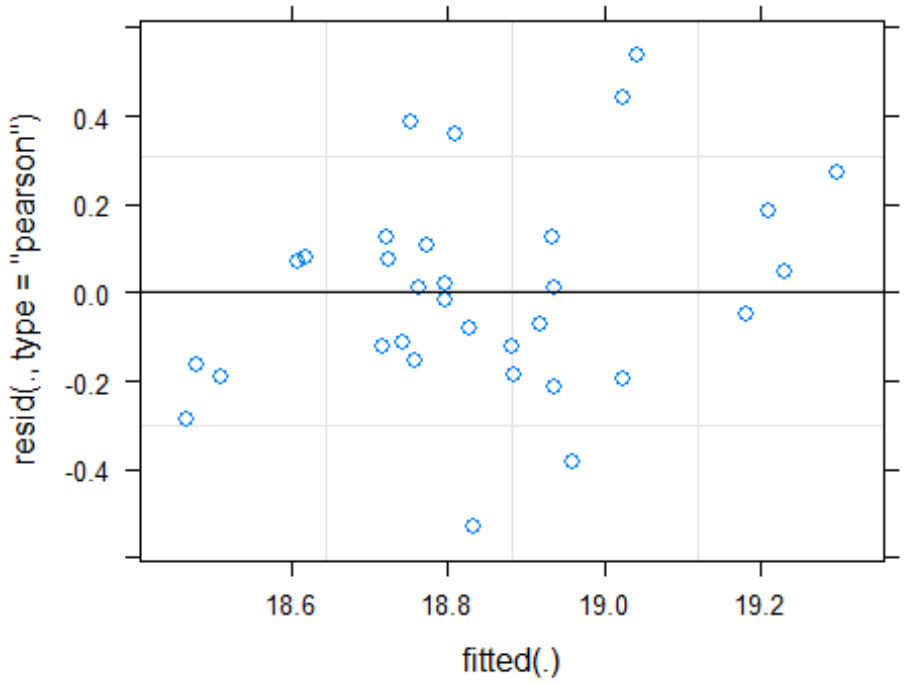
```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: B5X9M8_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
##     Stage + (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 24.1
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.88202 -0.54713 -0.01164  0.40165  1.91307
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.02653  0.1629
##  Week     (Intercept) 0.00000  0.0000
##  Residual             0.07970  0.2823
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error       df t value Pr(>|t|)
## (Intercept)         18.41657    0.19608 25.36400  93.925   <2e-16 ***
## rescale(VAP)         0.61029    0.24626 25.43800   2.478   0.0202 *
## rescale(SpermCount)  0.36422    0.24112 22.97000   1.511   0.1445
## StatusS             -0.16164    0.12007 26.78500  -1.346   0.1895
## StageB               0.01598    0.10326 10.88400   0.155   0.8798
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
##
## Correlation of Fixed Effects:
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.797
## rscl(SprmC) -0.581  0.306
## StatusS     -0.067 -0.165 -0.240
## StageB      -0.122 -0.096 -0.191  0.143
```

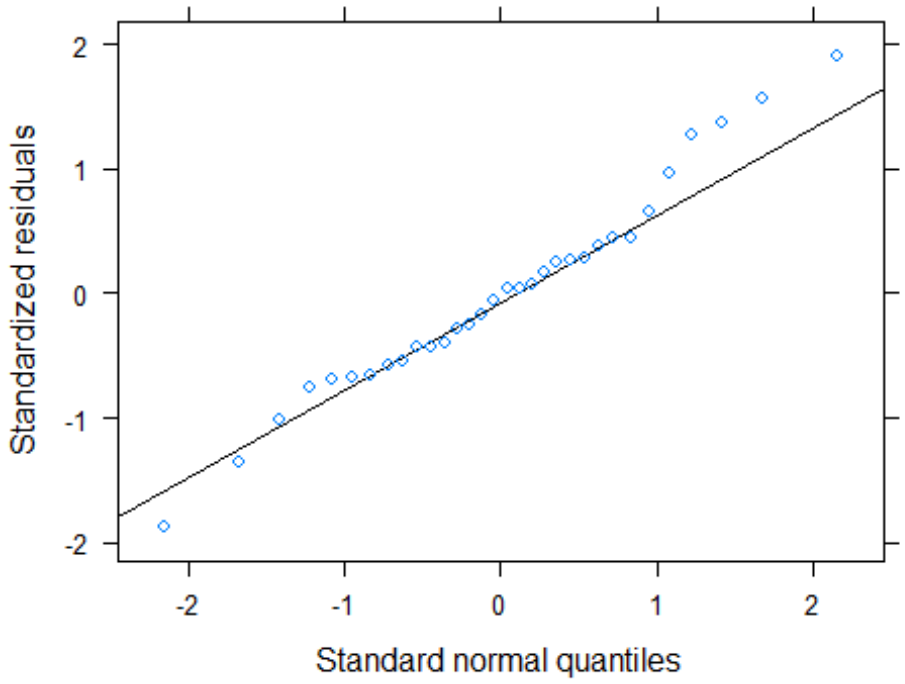
```
confint.merMod(modlist2[[137]],level=0.95,method="Wald")
```

```
##                      2.5 %      97.5 %
## .sig01                  NA          NA
## .sig02                  NA          NA
## .sigma                  NA          NA
## (Intercept) 15.7375362383 18.43553874
## VAP          0.0018103997  0.01550292
## SpermCount  -0.0003026801  0.00233744
## StageB      -0.1864113224  0.21837846
## StatusS     -0.3969778086  0.07369547
```

```
plot(modelP137, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```




```
qqmath(modelP137)#Visual Check Normality assumption
```



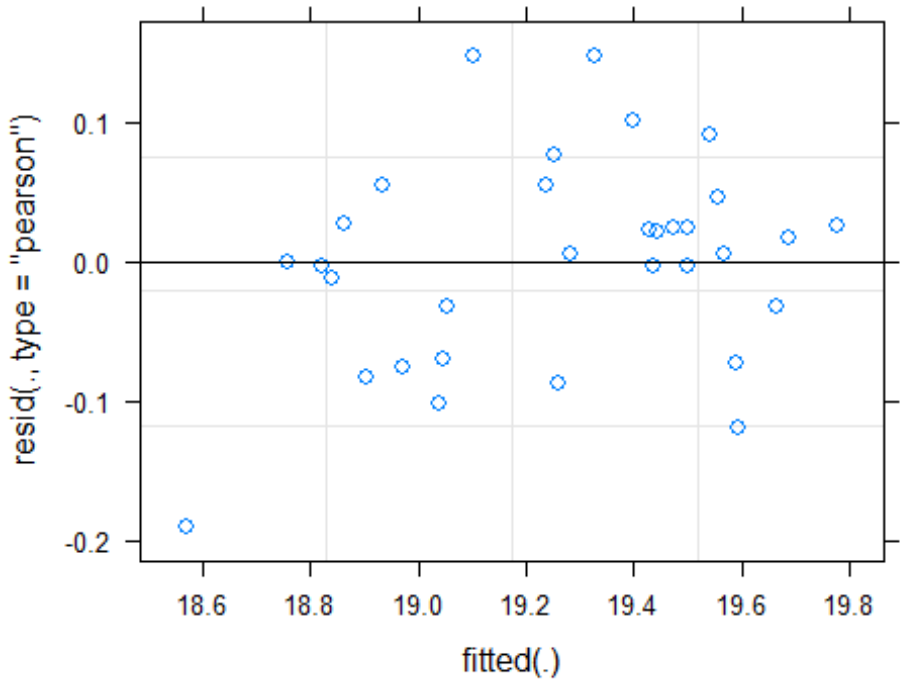

```
shapiro.test(resid(modelP137))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP137)
## W = 0.97783, p-value = 0.7345

rand(modelP137)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID  0.524      1     0.5
## Week    0.000      1     1.0

proteins[[153]]

## [1] "B5XBK1_SALSA"

modelP153<-lmer(B5XBK1_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
Stage + (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats)
summary(modelP153)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: B5XBK1_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
##     Stage + (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
```

```
##
## REML criterion at convergence: 12.1
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.61072 -0.35553  0.04323  0.26517  1.24777
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.11547  0.3398
##  Week     (Intercept) 0.00000  0.0000
##  Residual             0.01409  0.1187
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error       df t value Pr(>|t|)
## (Intercept)         18.85757    0.15006 25.15000 125.669  < 2e-16 ***
## rescale(VAP)         0.46759    0.16502 15.77300   2.833  0.01211 *
## rescale(SpermCount) -0.08698    0.12910 12.80000  -0.674  0.51245
## StatusS              0.18967    0.06786 12.77000   2.795  0.01539 *
## StageB               0.19284    0.04467 10.72600   4.318  0.00129 **
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.752
## rscl(SprmC) -0.549  0.460
## StatusS     -0.126 -0.048 -0.188
## StageB      -0.061 -0.093 -0.169  0.216
```

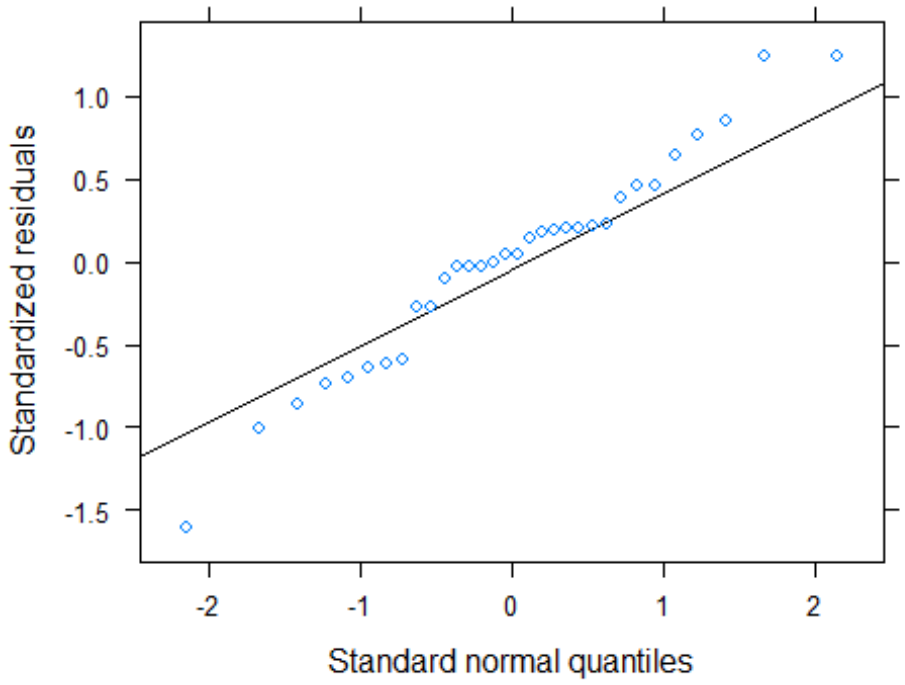
```
confint.merMod(modlist2[[153]],level=0.95,method="Wald")
```

```
##                     2.5 %       97.5 %
## .sig01                 NA           NA
## .sig02                 NA           NA
## .sigma                 NA           NA
## (Intercept) 17.1044302913 1.895096e+01
## VAP          0.0020446986 1.122031e-02
## SpermCount  -0.0009497461 4.638171e-04
## StageB       0.1053022174 2.803863e-01
## StatusS      0.0566731147 3.226642e-01
```

```
plot(modelP153, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```




```
qqmath(modelP153)#Visual Check Normality assumption
```




```
shapiro.test(resid(modelP153))#Test Check Normality assumption
```

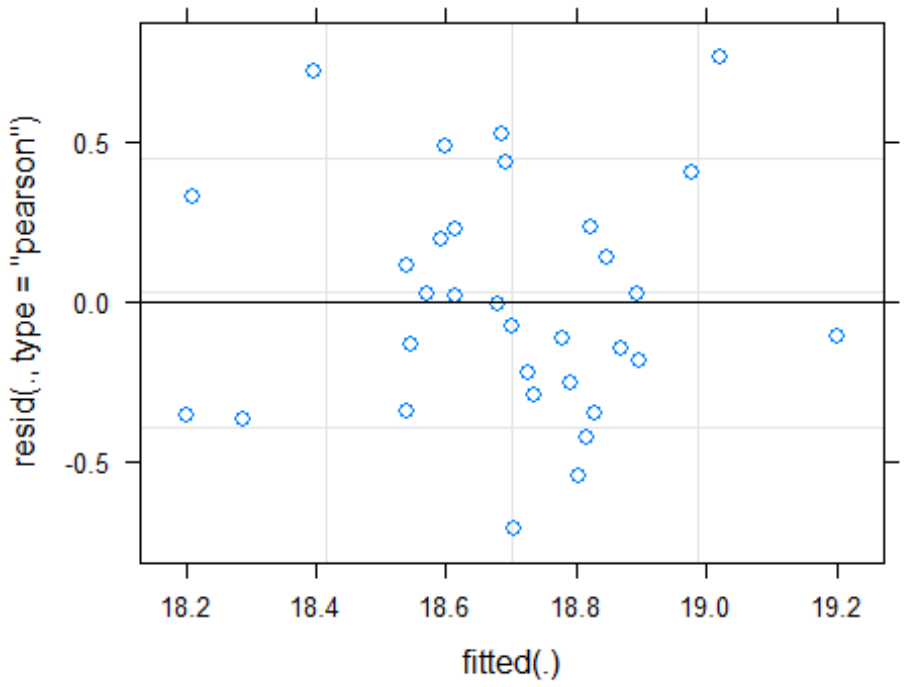
```
##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP153)
## W = 0.97234, p-value = 0.5664

rand(modelP153)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID   13.6      1   2e-04 ***
## Week      0.0      1       1
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

proteins[[156]]

## [1] "B5XBY3_SALSA"

modelP156<-lmer(B5XBY3_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
Stage + (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats)
summary(modelP153)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: B5XBK1_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
##     Stage + (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 12.1
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.61072 -0.35553  0.04323  0.26517  1.24777
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.11547  0.3398
##  Week     (Intercept) 0.00000  0.0000
##  Residual             0.01409  0.1187
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error       df t value Pr(>|t|)
## (Intercept)         18.85757    0.15006 25.15000 125.669  < 2e-16 ***
## rescale(VAP)         0.46759    0.16502 15.77300   2.833  0.01211 *
## rescale(SpermCount) -0.08698    0.12910 12.80000  -0.674  0.51245
## StatusS              0.18967    0.06786 12.77000   2.795  0.01539 *
## StageB               0.19284    0.04467 10.72600   4.318  0.00129 **
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
##
## Correlation of Fixed Effects:
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.752
## rscl(SprmC) -0.549  0.460
## StatusS     -0.126 -0.048 -0.188
## StageB      -0.061 -0.093 -0.169  0.216
```

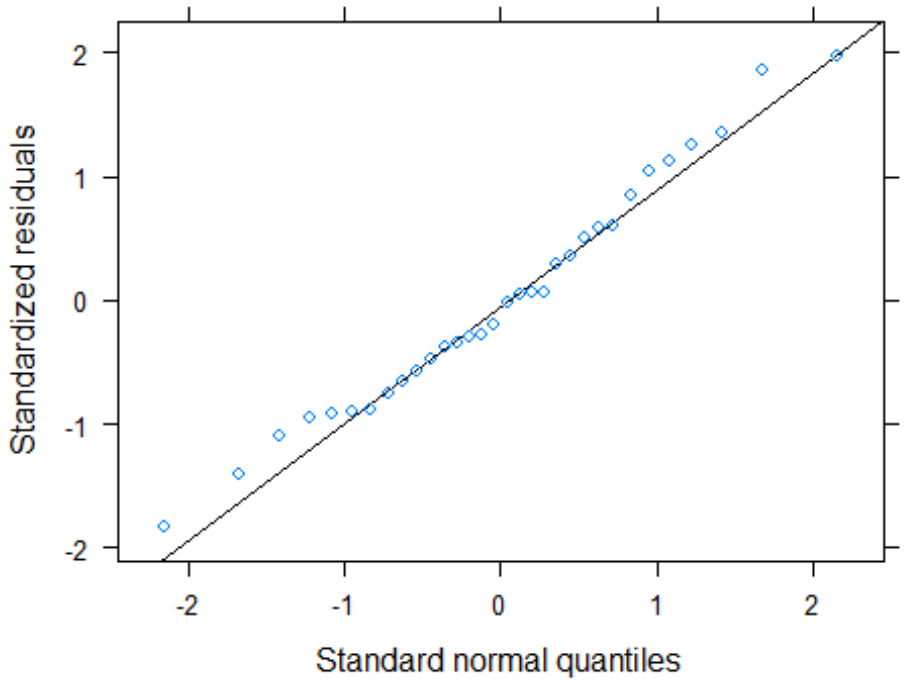
```
confint.merMod(modlist2[[156]],level=0.95,method="Wald")
```

```
##                      2.5 %       97.5 %
## .sig01                  NA           NA
## .sig02                  NA           NA
## .sigma                  NA           NA
## (Intercept) 15.2996603817 18.442100787
## VAP          0.0003785693  0.016330338
## SpermCount  -0.0010152955  0.002270654
## StageB      -0.0622011104  0.488559197
## StatusS     -0.1014790345  0.462679891
```

```
plot(modelP156, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```




```
qqmath(modelP156)#Visual Check Normality assumption
```



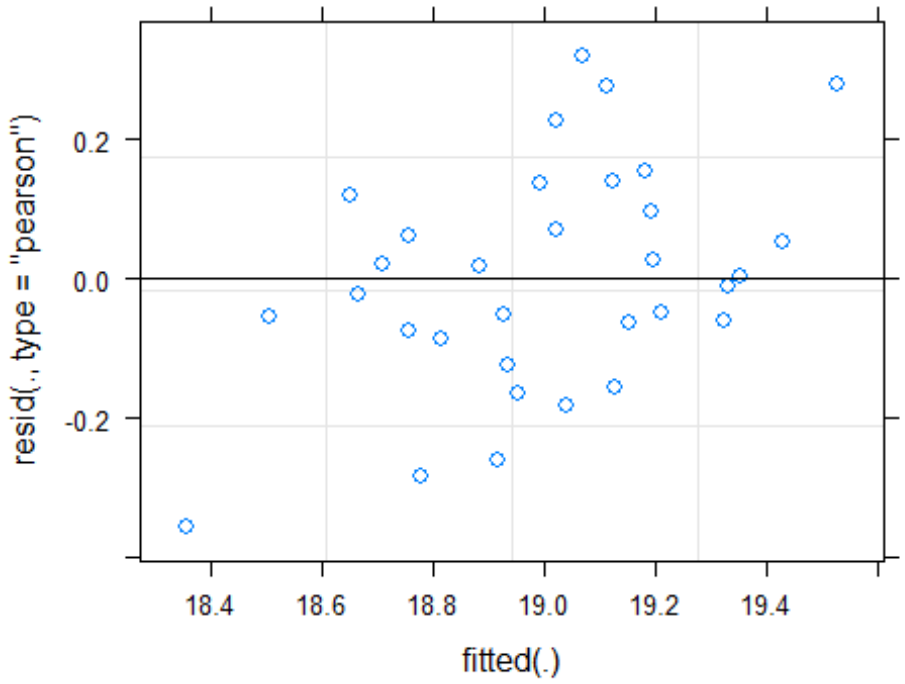

```
shapiro.test(resid(modelP156))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP156)
## W = 0.97943, p-value = 0.783

rand(modelP156)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID      0      1       1
## Week        0      1       1

proteins[[212]]

## [1] "B9EMK7_SALSA"

modelP212<-lmer(B9EMK7_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
Stage + (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats)
summary(modelP212)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: B9EMK7_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
##     Stage + (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
```

```
##
## REML criterion at convergence: 24.9
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.58245 -0.34407 -0.01293  0.45202  1.41954
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.07867  0.2805
##  Week     (Intercept) 0.00000  0.0000
##  Residual             0.05119  0.2263
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error       df t value Pr(>|t|)
## (Intercept)         18.48523    0.20310 26.97700  91.017   <2e-16 ***
## rescale(VAP)         0.52676    0.25051 26.08700   2.103   0.0453 *
## rescale(SpermCount)  0.41858    0.21978 19.26500   1.905   0.0719 .
## StatusS              0.17384    0.11397 21.18300   1.525   0.1420
## StageB              -0.06760    0.08401 12.92100  -0.805   0.4356
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.812
## rscl(SprmC) -0.582  0.375
## StatusS     -0.097 -0.115 -0.228
## StageB      -0.086 -0.099 -0.183  0.182
```

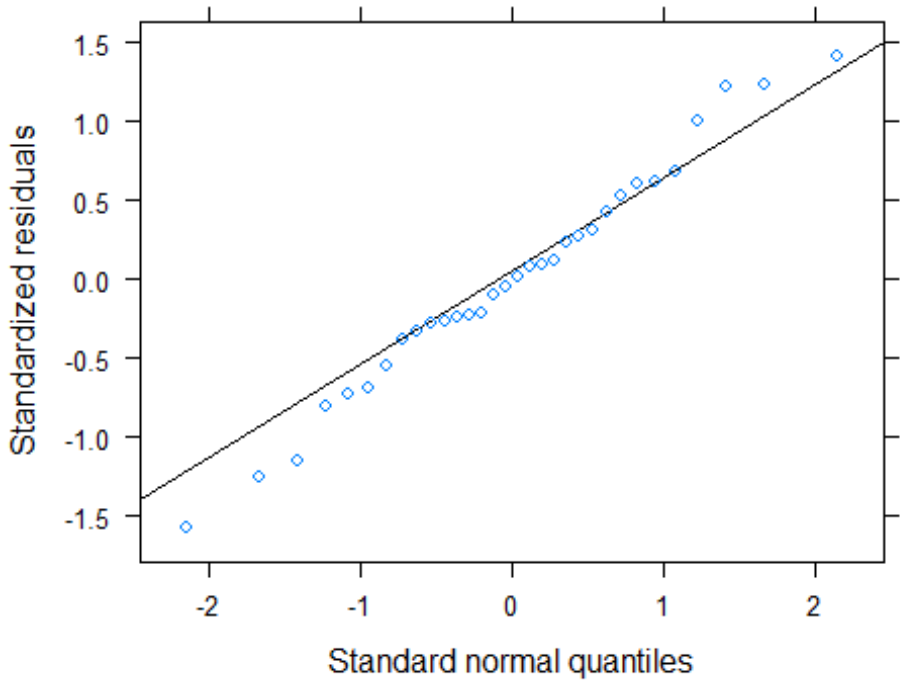
```
confint.merMod(modlist2[[212]],level=0.95,method="Wald")
```

```
##                     2.5 %       97.5 %
## .sig01                 NA           NA
## .sig02                 NA           NA
## .sigma                 NA           NA
## (Intercept)  1.590420e+01 18.662604406
## VAP          5.074640e-04  0.014436026
## SpermCount  -3.401002e-05  0.002372437
## StageB      -2.322454e-01  0.097054406
## StatusS     -4.953220e-02  0.397211288
```

```
plot(modelP212, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```




```
qqmath(modelP212)#Visual Check Normality assumption
```




```
shapiro.test(resid(modelP212))#Test Check Normality assumption
```

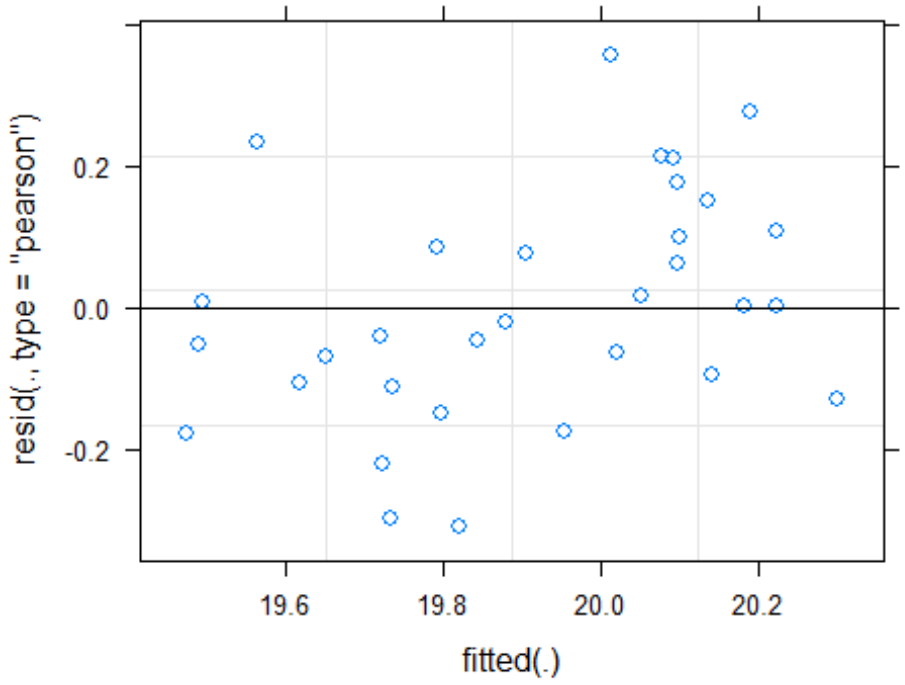
```
##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP212)
## W = 0.9859, p-value = 0.9413

rand(modelP212)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID   4.88      1    0.03 *
## Week     0.00      1    1.00
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

proteins[[231]]

## [1] "C0H7Q9_SALSA"

modelP231<-lmer(C0H7Q9_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
Stage + (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats)
summary(modelP231)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: C0H7Q9_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
##     Stage + (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 18.6
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.42544 -0.49669 -0.04326  0.46482  1.63794
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.04798  0.2190
##  Week     (Intercept) 0.00000  0.0000
##  Residual             0.04772  0.2185
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error       df t value Pr(>|t|)
## (Intercept)         20.33342    0.18058 26.90600 112.601   <2e-16 ***
## rescale(VAP)        -0.48070    0.22494 26.91400  -2.137   0.0418 *
## rescale(SpermCount) -0.33749    0.20437 20.11000  -1.651   0.1142
## StatusS              0.10794    0.10493 23.04600   1.029   0.3143
## StageB              -0.12249    0.08075 12.17300  -1.517   0.1548
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
##
## Correlation of Fixed Effects:
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.810
## rscl(SprmC) -0.582  0.352
## StatusS     -0.087 -0.132 -0.235
## StageB      -0.095 -0.099 -0.186  0.171
```

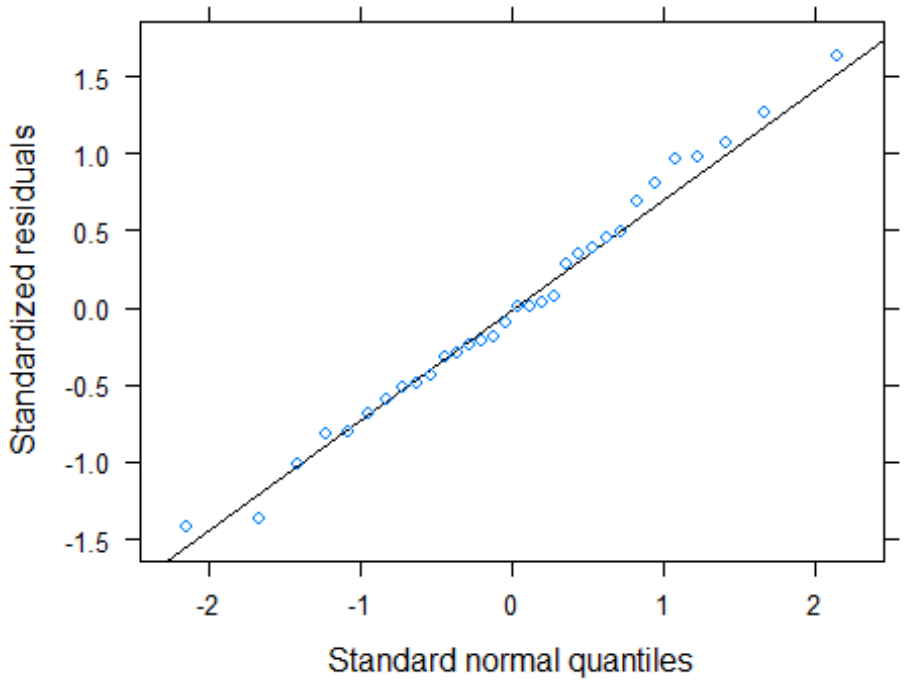
```
confint.merMod(modlist2[[231]],level=0.95,method="Wald")
```

```
##                     2.5 %        97.5 %
## .sig01                 NA            NA
## .sig02                 NA            NA
## .sigma                 NA            NA
## (Intercept) 20.171457513 22.6428872510
## VAP         -0.013071934 -0.0005649633
## SpermCount  -0.002061571  0.0001761273
## StageB      -0.280748079  0.0357697510
## StatusS     -0.097708678  0.3135980631
```

```
plot(modelP231, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```




```
qqmath(modelP231)#Visual Check Normality assumption
```



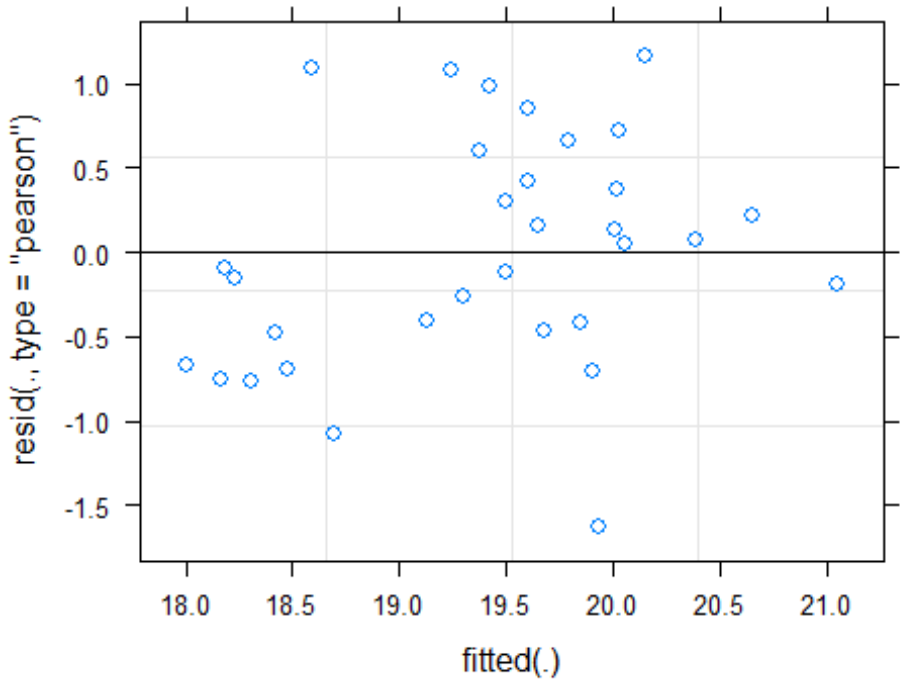

```
shapiro.test(resid(modelP231))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP231)
## W = 0.987, p-value = 0.9586

rand(modelP231)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID   2.86      1    0.09 .
## Week     0.00      1    1.00
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

proteins[[312]]

## [1] "Q0H913_SALSA"

modelP312<-lmer(Q0H913_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
Stage + (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats)
summary(modelP312)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: Q0H913_SALSA ~ rescale(VAP) + rescale(SpermCount) + Status +
```

```
##     Stage + (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 85.1
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.93029 -0.55443 -0.02989  0.55059  1.38389
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.27547  0.5249
##  Week     (Intercept) 0.06022  0.2454
##  Residual             0.71433  0.8452
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error       df t value Pr(>|t|)
## (Intercept)         19.82432    0.62580 20.41300  31.678   <2e-16 ***
## rescale(VAP)        -1.84708    0.78731 20.85600  -2.346   0.0289 *
## rescale(SpermCount)  0.83093    0.73464 23.50900   1.131   0.2694
## StatusS              0.02863    0.36516 23.65200   0.078   0.9382
## StageB               0.65280    0.30971 14.10200   2.108   0.0534 .
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.799
## rscl(SprmC) -0.574  0.323
## StatusS     -0.064 -0.156 -0.240
## StageB      -0.111 -0.096 -0.191  0.147
```

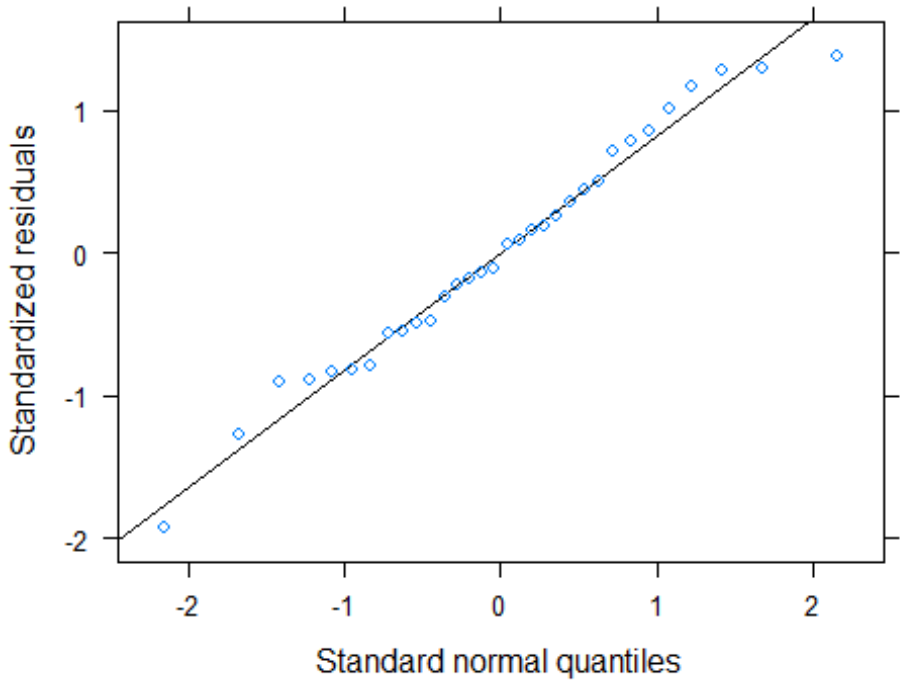
```
confint.merMod(modlist2[[312]],level=0.95,method="Wald")
```

```
##                    2.5 %       97.5 %
## .sig01                NA           NA
## .sig02                NA           NA
## .sigma                NA           NA
## (Intercept) 18.551112030 27.150243580
## VAP         -0.048087788 -0.004311744
## SpermCount  -0.001700959  0.006343014
## StageB       0.045777731  1.259830963
## StatusS     -0.687061318  0.744321364
```

```
plot(modelP312, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```




```
qqmath(modelP312)#Visual Check Normality assumption
```




```
shapiro.test(resid(modelP312))#Test Check Normality assumption
```

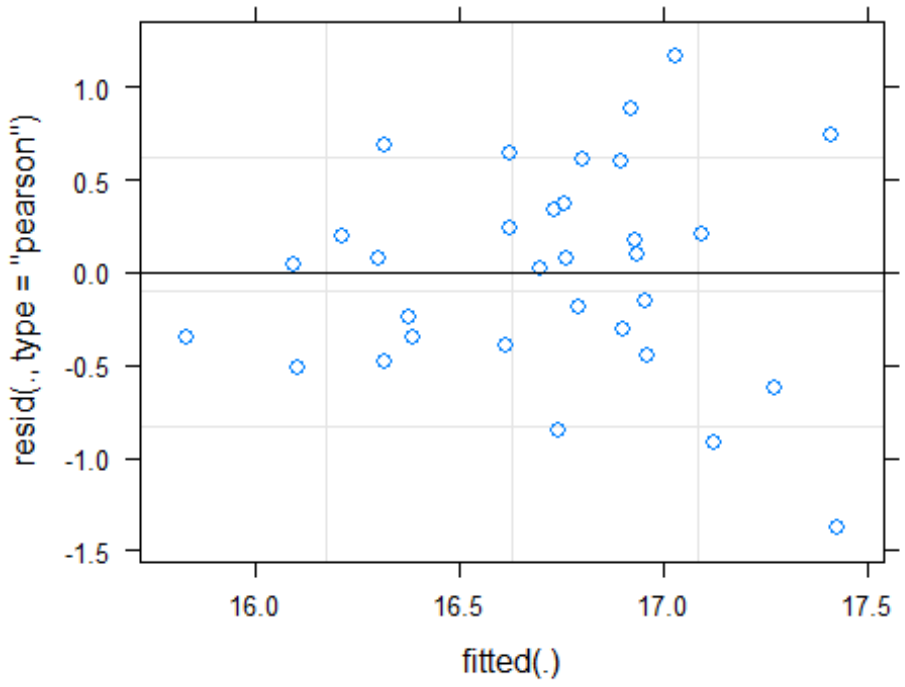
```
##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP312)
## W = 0.9773, p-value = 0.7181

rand(modelP312)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID 1.0035      1     0.3
## Week   0.0987      1     0.8

proteins[[321]]

## [1] "Q6R4A2_ONCMY"

modelP321<-lmer(Q6R4A2_ONCMY ~ rescale(VAP) + rescale(SpermCount) + Status +
Stage + (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats)
summary(modelP321)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula: Q6R4A2_ONCMY ~ rescale(VAP) + rescale(SpermCount) + Status +
##     Stage + (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 58.4
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -2.27596 -0.60091  0.09499  0.57099  1.93359
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.0000   0.0000
##  Week     (Intercept) 0.0000   0.0000
##  Residual             0.3675   0.6062
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error      df t value Pr(>|t|)
## (Intercept)          17.6130     0.3588 27.0000  49.090   <2e-16 ***
## rescale(VAP)         -0.9394     0.4482 27.0000  -2.096   0.0456 *
## rescale(SpermCount)  -1.4952     0.4688 27.0000  -3.189   0.0036 **
## StatusS               0.3735     0.2248 27.0000   1.661   0.1083
## StageB                0.1246     0.2195 27.0000   0.567   0.5751
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
```

```
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.776
## rscl(SprmC) -0.581  0.270
## StatusS     -0.053 -0.190 -0.235
## StageB      -0.158 -0.090 -0.191  0.117
```

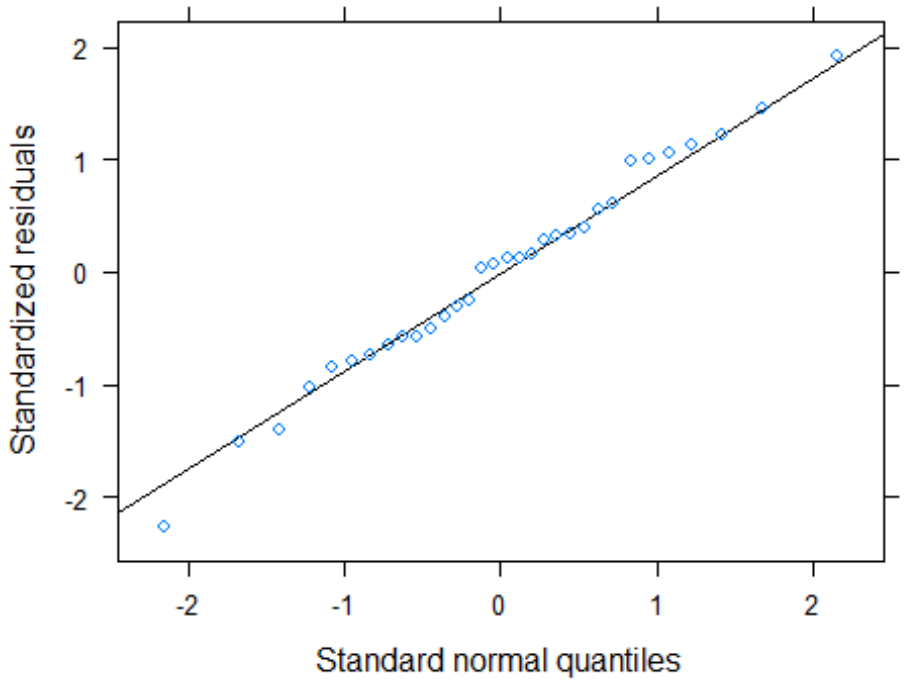
```
confint.merMod(modlist2[[321]],level=0.95,method="Wald")
```

```
##                    2.5 %        97.5 %
## .sig01                NA            NA
## .sig02                NA            NA
## .sigma                NA            NA
## (Intercept) 17.688531000 22.5977580925
## VAP         -0.025784469 -0.0008640735
## SpermCount  -0.006743223 -0.0016098015
## StageB      -0.305658148  0.5547583045
## StatusS     -0.067199923  0.8141483046
```

```
plot(modelP321, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```




```
qqmath(modelP321)#Visual Check Normality assumption
```




```
shapiro.test(resid(modelP321))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP321)
## W = 0.98957, p-value = 0.9858

rand(modelP321)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID      0      1       1
## Week        0      1       1
```

TESTING FOR A CORRELATION WITH SPERM NUMBER ACROSS MALES FROM BOTH STAGES: models with sperm number as significant predictor are shown below. Status and sperm velocity were included as fixed effects in these models, Experimental stage that ejaculates were collected is included as a cofactor

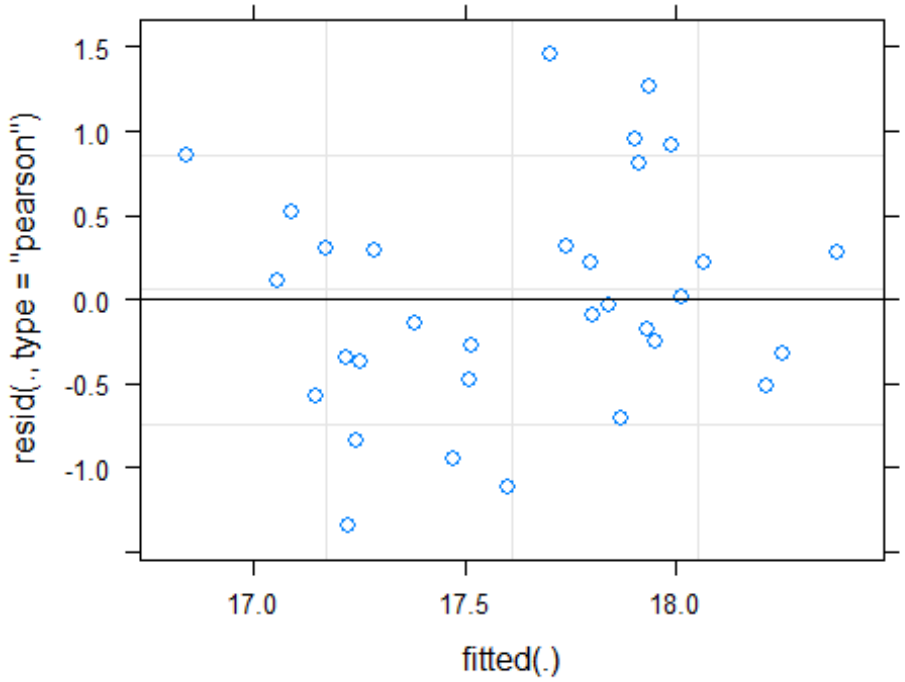
```
proteins[[8]]

## [1] "LYSC2_ONCMY"

modelP8<-lmer(LYSC2_ONCMY ~ rescale(VAP) + rescale(SpermCount) + Status + Stage + (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats)
summary(modelP8)
```

```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula:
## LYSC2_ONCMY ~ rescale(VAP) + rescale(SpermCount) + Status + Stage +
##     (1 | MaleID) + (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 73.1
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.77934 -0.52187 -0.08178  0.40393  1.92671
##
## Random effects:
##  Groups   Name        Variance  Std.Dev.
##  MaleID   (Intercept) 6.064e-02 2.463e-01
##  Week     (Intercept) 1.934e-15 4.398e-08
##  Residual             5.763e-01 7.592e-01
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##                     Estimate Std. Error      df t value Pr(>|t|)
## (Intercept)          18.4620     0.4774 25.0700  38.670   <2e-16 ***
## rescale(VAP)         -0.3770     0.5985 24.2770  -0.630   0.5346
## rescale(SpermCount)  -1.4641     0.6108 25.7850  -2.397   0.0241 *
## StatusS               0.1521     0.2974 26.6880   0.511   0.6133
## StageB               -0.2274     0.2759 13.7580  -0.824   0.4239
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) r(VAP) rs(SC) StatsS
## rescal(VAP) -0.785
## rscl(SprmC) -0.581  0.283
## StatusS     -0.058 -0.181 -0.238
## StageB      -0.143 -0.093 -0.191  0.127
```

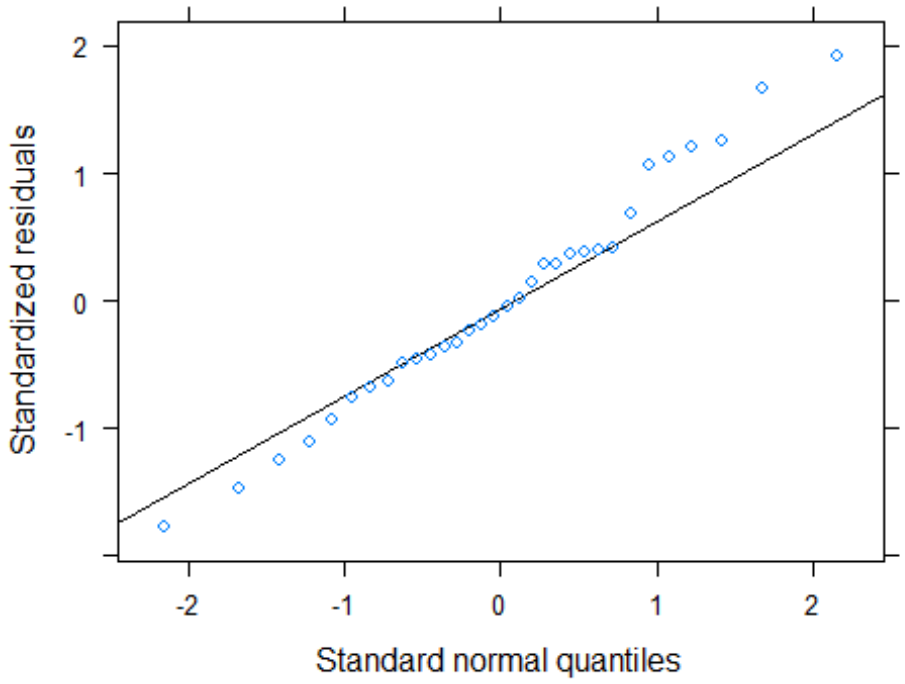
```
confint.merMod(modlist2[[8]],level=0.95,method="Wald")
```

```
##                     2.5 %        97.5 %
## .sig01                 NA            NA
## .sig02                 NA            NA
## .sigma                 NA            NA
## (Intercept) 16.646984018 23.2008286126
## VAP         -0.021985317  0.0112901101
## SpermCount  -0.007433651 -0.0007455185
## StageB      -0.768251801  0.3134436453
## StatusS     -0.430822228  0.7349920741
```

```
plot(modelP8, results="hide", fig.show='hide')#Visual Check Variance assumpti
on
```




```
qqmath(modelP8)#Visual Check Normality assumption
```



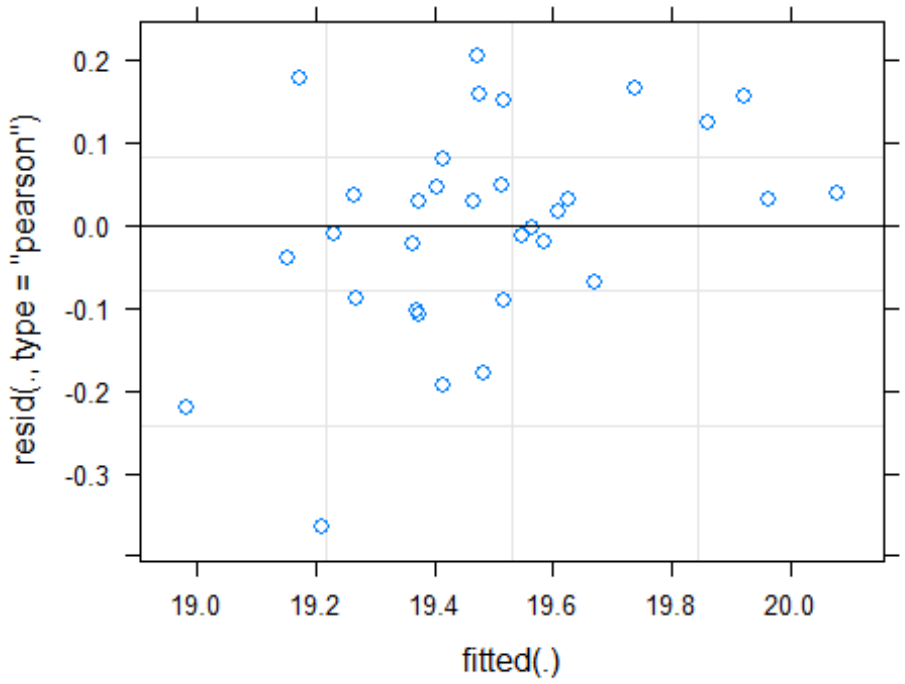

```
shapiro.test(resid(modelP8))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP8)
## W = 0.98557, p-value = 0.9354

rand(modelP8)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID  0.109      1     0.7
## Week    0.000      1     1.0

proteins[[22]]

## [1] "B2DBF2_ONCMY"

modelP22<-lmer(B2DBF2_ONCMY ~ VAP + SpermCount + Status + Stage +
                (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats)
summary(modelP22)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula:
## B2DBF2_ONCMY ~ VAP + SpermCount + Status + Stage + (1 | MaleID) +
##     (1 | Week)
```

```
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 36.9
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -2.0050 -0.4036  0.1231  0.3125  1.1289
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.05758  0.2400
##  Week     (Intercept) 0.01625  0.1275
##  Residual             0.03329  0.1825
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##               Estimate Std. Error         df t value Pr(>|t|)
## (Intercept)  1.901e+01  6.127e-01  2.150e+01  31.019   <2e-16 ***
## VAP         -9.339e-05  3.095e-03  2.015e+01  -0.030   0.9762
## SpermCount   1.371e-03  5.054e-04  1.289e+01   2.712   0.0179 *
## StatusS      5.095e-02  9.319e-02  1.488e+01   0.547   0.5927
## StageB       1.061e-01  6.787e-02  7.384e+00   1.563   0.1598
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn StatsS
## VAP         -0.961
## SpermCount -0.587  0.401
## StatusS     0.072 -0.100 -0.222
## StageB      0.071 -0.098 -0.182  0.186
```

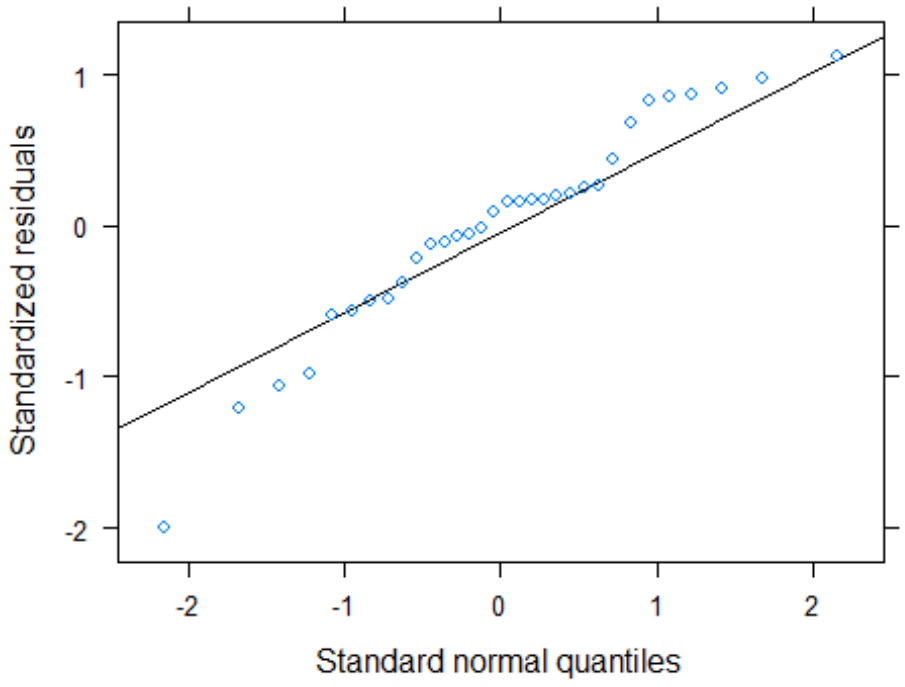
```
confint.merMod(modlist2[[22]],level=0.95,method="Wald")
```

```
##                      2.5 %       97.5 %
## .sig01                  NA           NA
## .sig02                  NA           NA
## .sigma                  NA           NA
## (Intercept) 17.8047431145 20.206487194
## VAP         -0.0061586958  0.005971925
## SpermCount   0.0003801308  0.002361309
## StageB      -0.0269310468  0.239130788
## StatusS     -0.1316941533  0.233593289
```

```
plot(modelP22, results="hide", fig.show='hide')#Visual Check Variance assumpt
ion
```




```
qqmath(modelP22)#Visual Check Normality assumption
```




```
shapiro.test(resid(modelP22))#Test Check Normality assumption
```

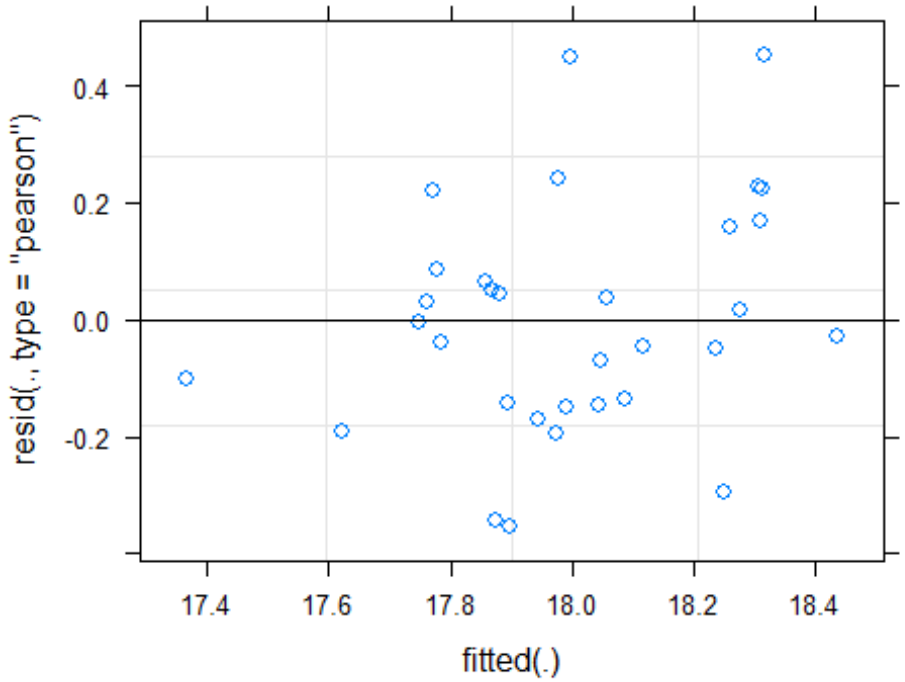
```
##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP22)
## W = 0.95204, p-value = 0.1646

rand(modelP22)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID  3.226      1    0.07 .
## Week    0.453      1    0.50
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

proteins[[81]]

## [1] "B5X2Q5_SALSA"

modelP81<-lmer(B5X2Q5_SALSA ~ VAP + SpermCount + Status + Stage +
                 (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats)
summary(modelP81)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula:
## B5X2Q5_SALSA ~ VAP + SpermCount + Status + Stage + (1 | MaleID) +
##     (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 41
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.4414 -0.5852 -0.0651  0.4188  1.8405
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.01669  0.1292
##  Week     (Intercept) 0.04217  0.2054
##  Residual             0.06079  0.2466
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##               Estimate Std. Error         df t value Pr(>|t|)
## (Intercept) 20.5151894  0.6891946 26.9710000  29.767  < 2e-16 ***
## VAP         -0.0118260  0.0035197 26.9530000  -3.360  0.00234 **
## SpermCount  -0.0016368  0.0005955 23.0070000  -2.749  0.01143 *
## StatusS     -0.0494158  0.1035112 22.7830000  -0.477  0.63763
## StageB       0.1366467  0.0902763 14.1280000   1.514  0.15216
## ---
```

```
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn StatsS
## VAP         -0.955
## SpermCount -0.553  0.346
## StatusS     0.126 -0.150 -0.240
## StageB      0.066 -0.094 -0.195  0.141
```

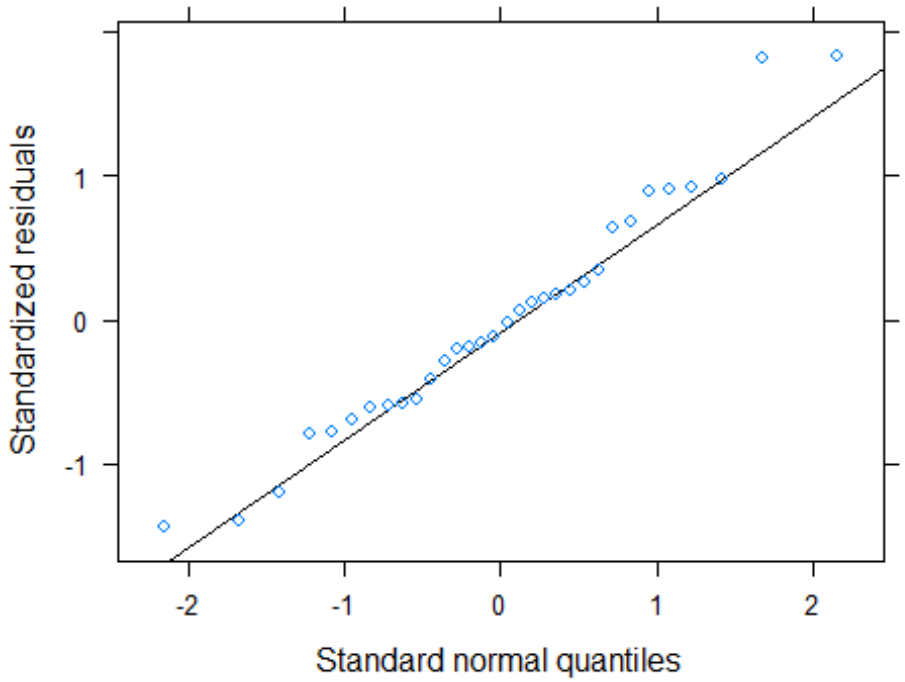
```
confint.merMod(modlist2[[81]],level=0.95,method="Wald")
```

```
##                     2.5 %        97.5 %
## .sig01                 NA            NA
## .sig02                 NA            NA
## .sigma                 NA            NA
## (Intercept) 19.164392685 21.8659860586
## VAP         -0.018724545 -0.0049274110
## SpermCount  -0.002803879 -0.0004697342
## StageB      -0.040291499  0.3135849369
## StatusS     -0.252293991  0.1534623380
```

```
plot(modelP81, results="hide", fig.show='hide')#Visual Check Variance assumpt
ion
```




```
qqmath(modelP81)#Visual Check Normality assumption
```



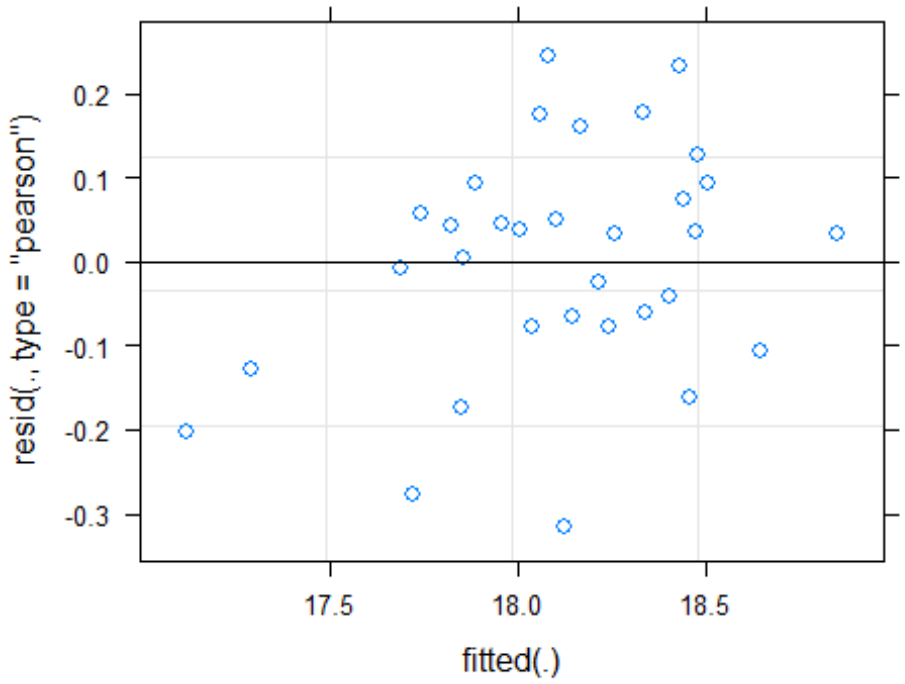

```
shapiro.test(resid(modelP81))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP81)
## W = 0.96898, p-value = 0.4719

rand(modelP81)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID  0.558      1     0.5
## Week    2.638      1     0.1

proteins[[88]]

## [1] "B5X3I8_SALSA"

modelP88<-lmer(B5X3I8_SALSA ~ VAP + SpermCount + Status + Stage +
                 (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats)
summary(modelP88)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula:
## B5X3I8_SALSA ~ VAP + SpermCount + Status + Stage + (1 | MaleID) +
##     (1 | Week)
```

```
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 44.2
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.5842 -0.3838  0.1634  0.3959  1.2299
##
## Random effects:
##  Groups   Name        Variance  Std.Dev.
##  MaleID   (Intercept) 9.965e-02 3.157e-01
##  Week     (Intercept) 5.155e-15 7.180e-08
##  Residual             3.995e-02 1.999e-01
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##               Estimate Std. Error         df t value Pr(>|t|)
## (Intercept) 18.1607706  0.6755023 23.6340000  26.885  < 2e-16 ***
## VAP         -0.0044432  0.0033996 22.9310000  -1.307  0.20418
## SpermCount   0.0019135  0.0005646 15.4380000   3.389  0.00391 **
## StatusS     -0.0190367  0.1056518 16.5390000  -0.180  0.85920
## StageB       0.2732961  0.0745757 10.5000000   3.665  0.00402 **
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn StatsS
## VAP         -0.963
## SpermCount -0.592  0.403
## StatusS     0.064 -0.093 -0.217
## StageB      0.070 -0.098 -0.179  0.195
```

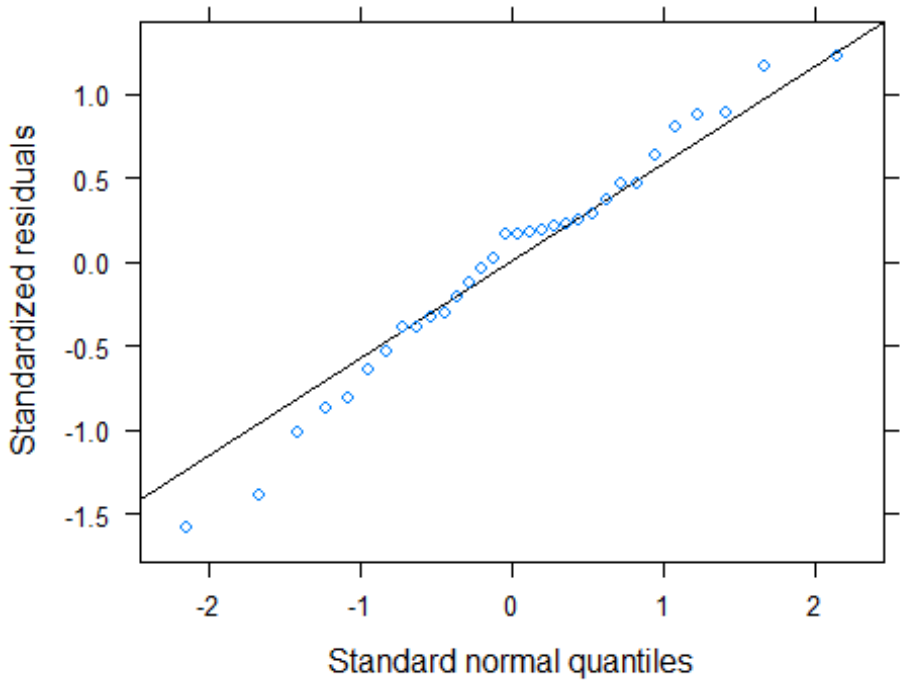
```
confint.merMod(modlist2[[88]],level=0.95,method="Wald")
```

```
##                      2.5 %       97.5 %
## .sig01                  NA           NA
## .sig02                  NA           NA
## .sigma                  NA           NA
## (Intercept) 16.8368104379 19.484730741
## VAP         -0.0111063764  0.002219942
## SpermCount   0.0008068808  0.003020034
## StageB       0.1271304084  0.419461711
## StatusS     -0.2261103827  0.188037047
```

```
plot(modelP88, results="hide", fig.show='hide')#Visual Check Variance assumpt
ion
```




```
qqmath(modelP88)#Visual Check Normality assumption
```




```
shapiro.test(resid(modelP88))#Test Check Normality assumption
```

```
##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP88)
## W = 0.97808, p-value = 0.7422
```

```
rand(modelP88)#Test for significance of random predictor male ID
```

```
## Analysis of Random effects Table:
##          Chi.sq Chi.DF p.value
## MaleID 6.47e+00      1    0.01 *
## Week   2.20e-13      1    1.00
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
proteins[[90]]
```

```
## [1] "B5X3P8_SALSA"
```

```
modelP90<-lmer(B5X3P8_SALSA ~ VAP + SpermCount + Status + Stage +
                 (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats)
summary(modelP90)
```

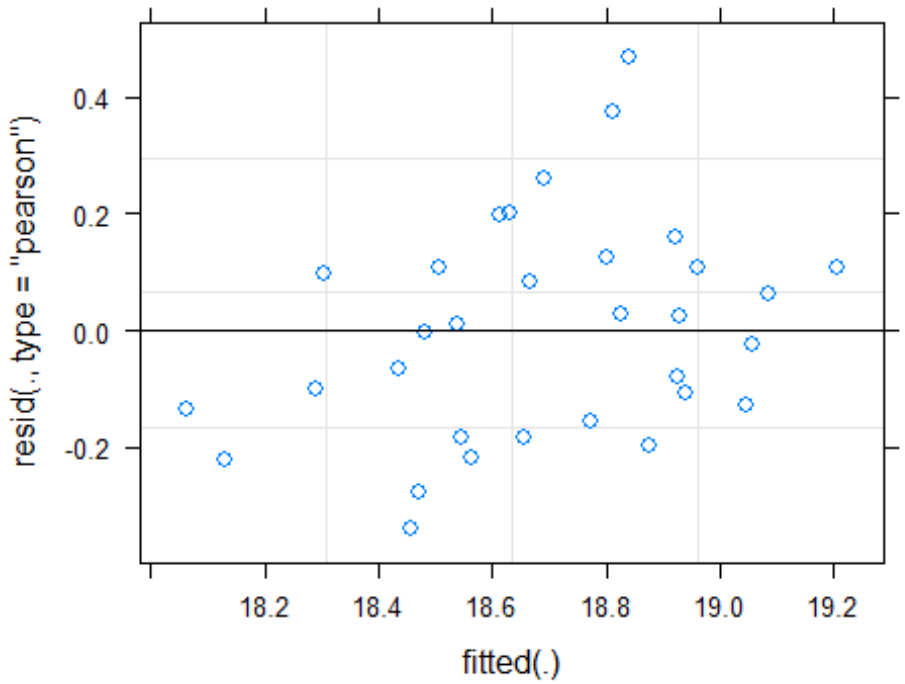
```
## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula:
## B5X3P8_SALSA ~ VAP + SpermCount + Status + Stage + (1 | MaleID) +
##     (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 42.4
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.3975 -0.5753  0.0192  0.4440  1.9243
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.04569  0.2138
##  Week     (Intercept) 0.00000  0.0000
##  Residual             0.05983  0.2446
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##               Estimate Std. Error         df t value Pr(>|t|)
## (Intercept) 19.6931694  0.6735307 26.9340000  29.239  < 2e-16 ***
## VAP         -0.0021403  0.0034121 26.9830000  -0.627  0.53577
## SpermCount  -0.0017888  0.0006237 21.4240000  -2.868  0.00909 **
## StatusS      0.0758975  0.1137941 24.6200000   0.667  0.51099
## StageB      -0.1875631  0.0901464 12.6960000  -2.081  0.05831 .
## ---
```

```
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn StatsS
## VAP         -0.957
## SpermCount -0.563  0.338
## StatusS     0.112 -0.142 -0.237
## StageB      0.069 -0.099 -0.188  0.163
```

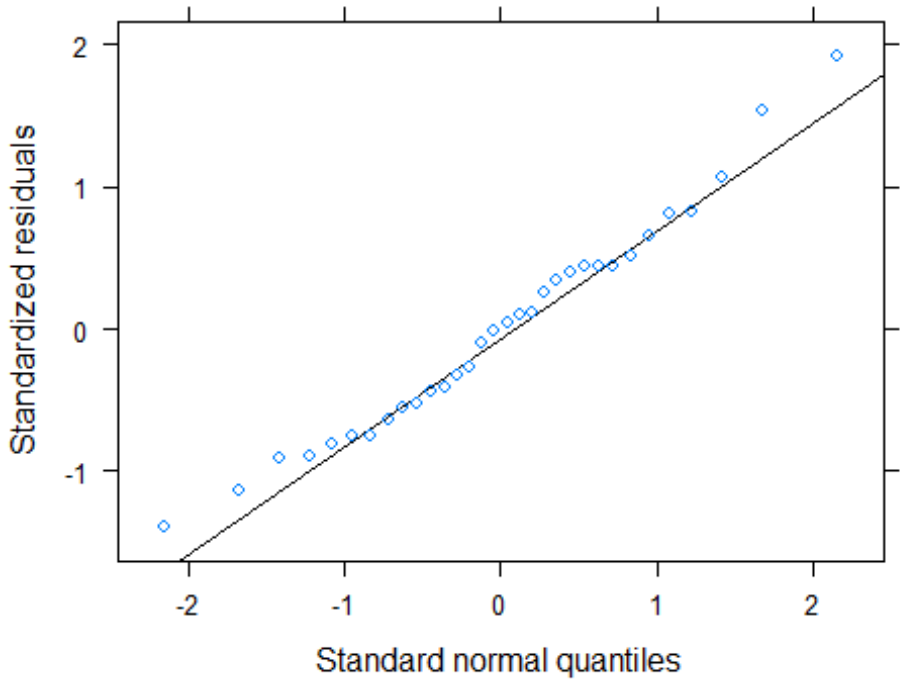
```
confint.merMod(modelP90,level=0.95,method="Wald")
```

```
##                     2.5 %        97.5 %
## .sig01                 NA            NA
## .sig02                 NA            NA
## .sigma                 NA            NA
## (Intercept) 18.373073549 21.0132653285
## VAP         -0.008827944  0.0045474004
## SpermCount  -0.003011266 -0.0005662909
## StatusS     -0.147134944  0.2989298877
## StageB      -0.364246776 -0.0108794411
```

```
plot(modelP90, results="hide", fig.show='hide')#Visual Check Variance assumption
```




```
qqmath(modelP90)#Visual Check Normality assumption
```



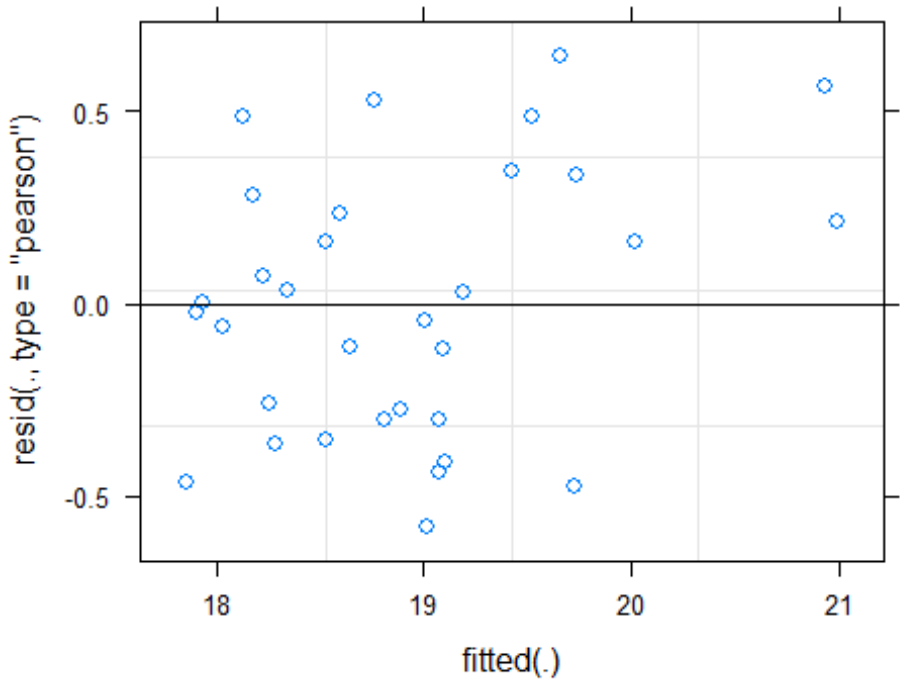

```
shapiro.test(resid(modelP90))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP90)
## W = 0.97672, p-value = 0.7003

rand(modelP90)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##          Chi.sq Chi.DF p.value
## MaleID 2.26e+00      1     0.1
## Week   7.11e-14      1     1.0

proteins[[99]]

## [1] "B5X4I3_SALSA"

modelP99<-lmer(B5X4I3_SALSA ~ VAP + SpermCount + Status + Stage +
                 (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats)
summary(modelP99)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula:
## B5X4I3_SALSA ~ VAP + SpermCount + Status + Stage + (1 | MaleID) +
##     (1 | Week)
```

```
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 88.4
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.20322 -0.61888 -0.02196  0.50992  1.33541
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.2754   0.5248
##  Week     (Intercept) 0.3414   0.5843
##  Residual             0.2341   0.4839
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##              Estimate Std. Error        df t value Pr(>|t|)
## (Intercept) 20.043942   1.620524 25.069000  12.369  3.6e-12 ***
## VAP         -0.002127   0.008175 23.973000  -0.260   0.7970
## SpermCount  -0.002969   0.001310 19.354000  -2.267   0.0350 *
## StatusS      0.552817   0.237347 21.369000   2.329   0.0297 *
## StageB      -0.083347   0.179430 12.746000  -0.465   0.6501
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn StatsS
## VAP         -0.955
## SpermCount -0.578  0.398
## StatusS     0.081 -0.107 -0.227
## StageB      0.071 -0.097 -0.187  0.176
```

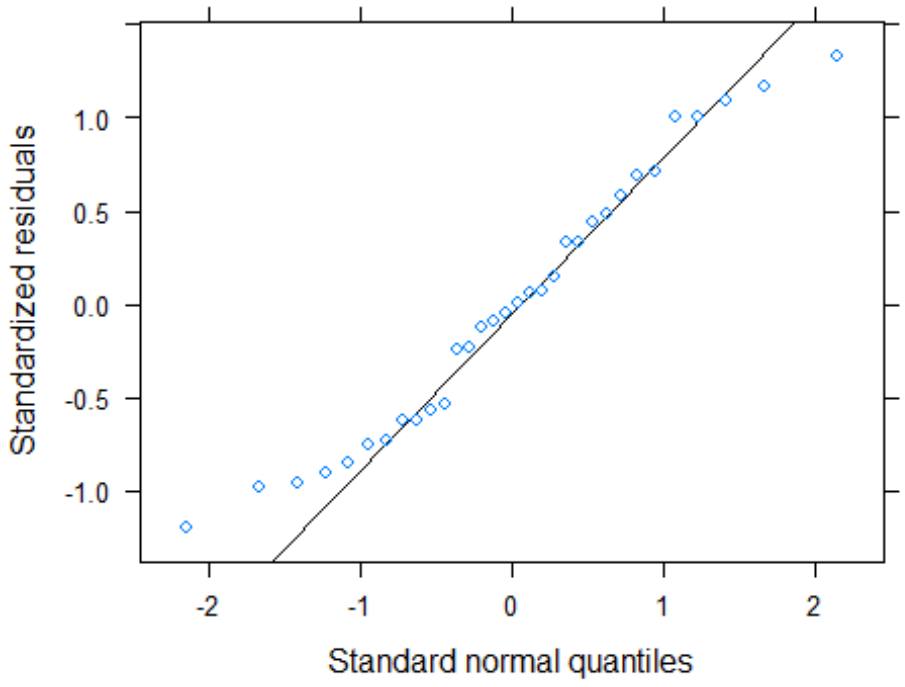
```
confint.merMod(modelP99,level=0.95,method="Wald")
```

```
##                    2.5 %        97.5 %
## .sig01                NA            NA
## .sig02                NA            NA
## .sigma                NA            NA
## (Intercept) 16.867773410 23.2201097630
## VAP         -0.018149677  0.0138961142
## SpermCount  -0.005535386 -0.0004019461
## StatusS      0.087625386  1.0180077883
## StageB      -0.435023398  0.2683286652
```

```
plot(modelP99, results="hide", fig.show='hide')#Visual Check Variance assumpt
ion
```




```r
qqmath(modelP99)#Visual Check Normality assumption
```




```r
shapiro.test(resid(modelP99))#Test Check Normality assumption
```

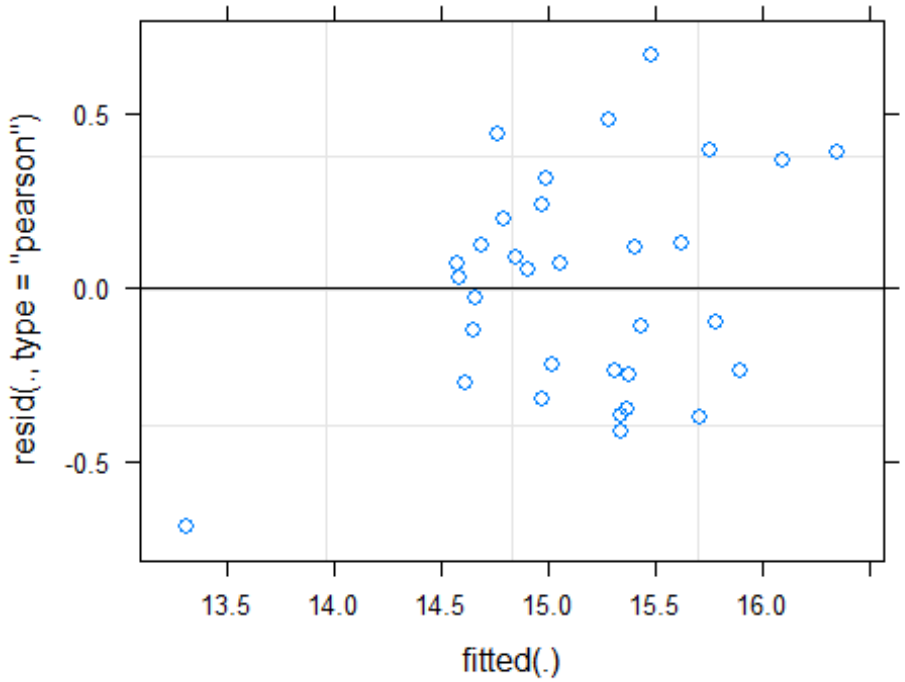
```
##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP99)
## W = 0.96194, p-value = 0.3101

rand(modelP99)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID   3.87      1    0.05 *
## Week     3.53      1    0.06 .
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

proteins[[116]]

## [1] "B5X6Y1_SALSA"

modelP116<-lmer(B5X6Y1_SALSA ~ VAP + SpermCount + Status + Stage +
                  (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats)
summary(modelP116)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula:
## B5X6Y1_SALSA ~ VAP + SpermCount + Status + Stage + (1 | MaleID) +
##     (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 74.8
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.64186 -0.58403  0.08705  0.48756  1.58767
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.15483  0.3935
##  Week     (Intercept) 0.04936  0.2222
##  Residual             0.17738  0.4212
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##              Estimate Std. Error        df t value Pr(>|t|)
## (Intercept) 15.666454   1.265988 25.160000  12.375 3.38e-12 ***
## VAP          0.004698   0.006431 24.534000   0.731  0.47196
## SpermCount  -0.003159   0.001099 20.681000  -2.875  0.00916 **
## StatusS     -0.111945   0.199333 22.544000  -0.562  0.57993
## StageB      -0.541314   0.155575 12.754000  -3.479  0.00418 **
## ---
```

```
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn StatsS
## VAP         -0.960
## SpermCount -0.570  0.365
## StatusS     0.101 -0.128 -0.234
## StageB      0.070 -0.097 -0.188  0.167
```

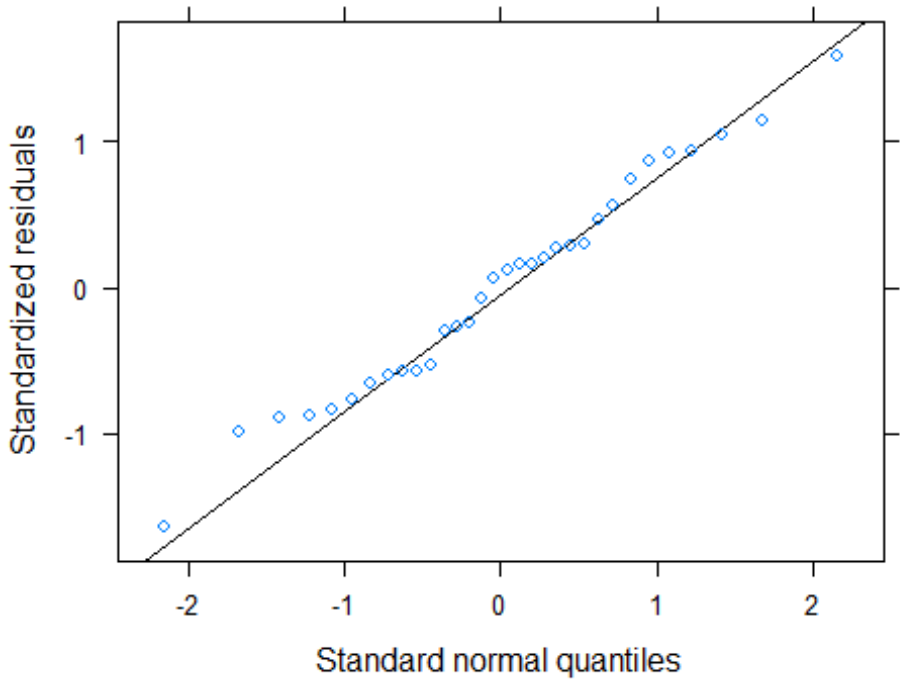
```
confint.merMod(modelP116,level=0.95,method="Wald")
```

```
##                    2.5 %       97.5 %
## .sig01                NA           NA
## .sig02                NA           NA
## .sigma                NA           NA
## (Intercept) 13.185162520 18.147745986
## VAP         -0.007905670  0.017301648
## SpermCount  -0.005312894 -0.001005152
## StatusS     -0.502631392  0.278740906
## StageB      -0.846235958 -0.236392830
```

```
plot(modelP116, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```




```
qqmath(modelP116)#Visual Check Normality assumption
```



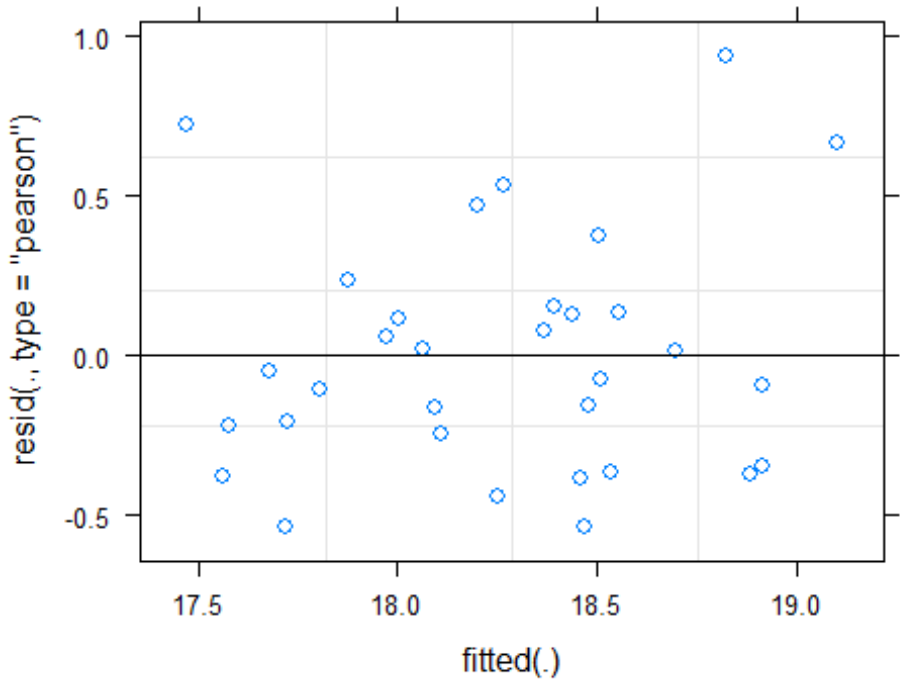

```
shapiro.test(resid(modelP116))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP116)
## W = 0.98164, p-value = 0.8456

rand(modelP116)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID  2.657      1     0.1
## Week    0.398      1     0.5

proteins[[123]]

## [1] "B5X834_SALSA"

modelP123<-lmer(B5X834_SALSA ~ VAP + SpermCount + Status + Stage +
                  (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats
)
summary(modelP123)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula:
## B5X834_SALSA ~ VAP + SpermCount + Status + Stage + (1 | MaleID) +
```

```
##     (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 68.3
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.2579 -0.6235 -0.1408  0.3294  2.1980
##
## Random effects:
##  Groups   Name        Variance  Std.Dev.
##  MaleID   (Intercept) 2.470e-15 4.970e-08
##  Week     (Intercept) 2.427e-01 4.927e-01
##  Residual             1.834e-01 4.283e-01
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##               Estimate Std. Error         df t value Pr(>|t|)
## (Intercept) 19.7166214  1.1186114 26.6110000  17.626 4.44e-16 ***
## VAP         -0.0051651  0.0056903 25.6950000  -0.908   0.3725
## SpermCount  -0.0021413  0.0009586 23.4370000  -2.234   0.0353 *
## StatusS      0.0680644  0.1600318 23.0260000   0.425   0.6746
## StageB       0.1161197  0.1556313 22.9760000   0.746   0.4632
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn StatsS
## VAP         -0.944
## SpermCount -0.536  0.331
## StatusS     0.144 -0.169 -0.237
## StageB      0.060 -0.090 -0.199  0.119
```

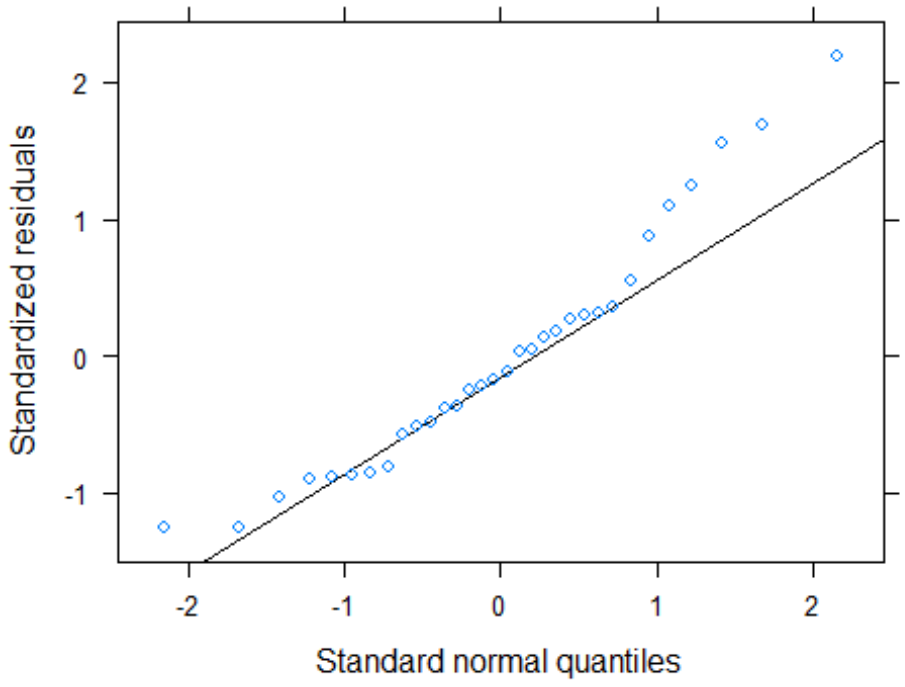
```
confint.merMod(modelP123,level=0.95,method="Wald")
```

```
##                    2.5 %        97.5 %
## .sig01                NA            NA
## .sig02                NA            NA
## .sigma                NA            NA
## (Intercept) 17.524183435 21.9090594296
## VAP         -0.016317798  0.0059876749
## SpermCount  -0.004020078 -0.0002625585
## StatusS     -0.245592135  0.3817208815
## StageB      -0.188912048  0.4211514669
```

```
plot(modelP123, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```




```
qqmath(modelP123)#Visual Check Normality assumption
```




```
shapiro.test(resid(modelP123))#Test Check Normality assumption
```

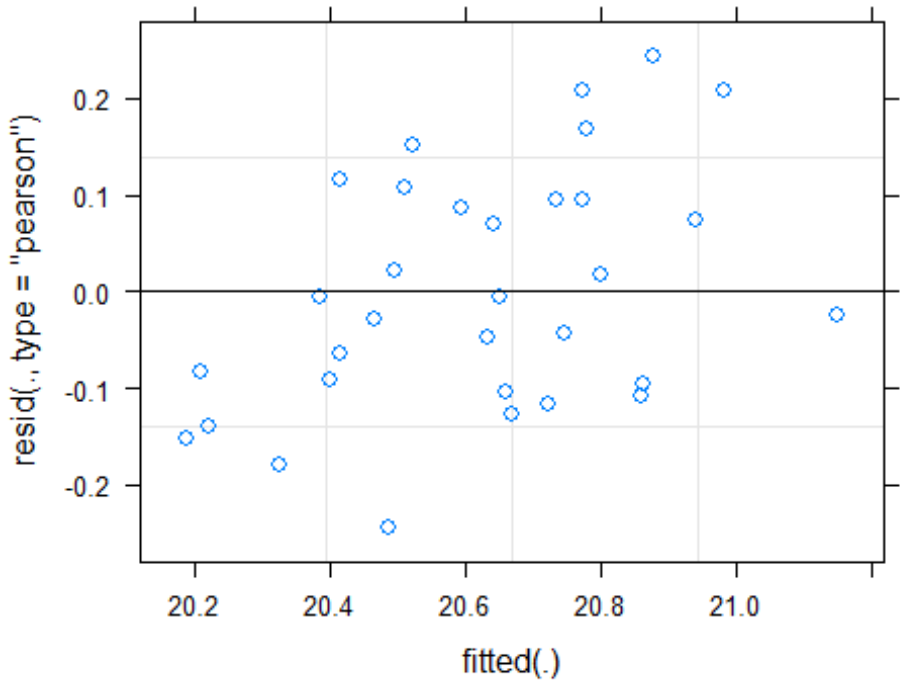
```
##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP123)
## W = 0.9461, p-value = 0.1116

rand(modelP123)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID   0.00      1   1.000
## Week     8.56      1   0.003 **
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

proteins[[161]]

## [1] "B5XCB2_SALSA"

modelP161<-lmer(B5XCB2_SALSA ~ VAP + SpermCount + Status + Stage +
                  (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats
)
summary(modelP161)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula:
## B5XCB2_SALSA ~ VAP + SpermCount + Status + Stage + (1 | MaleID) +
##     (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 36.3
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.36931 -0.54564 -0.08805  0.53120  1.36438
##
## Random effects:
##  Groups   Name        Variance  Std.Dev.
##  MaleID   (Intercept) 6.793e-02 0.260633
##  Week     (Intercept) 5.384e-05 0.007338
##  Residual             3.210e-02 0.179164
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##               Estimate Std. Error         df t value Pr(>|t|)
## (Intercept) 20.1025333  0.5892150 19.9840000  34.117  < 2e-16 ***
## VAP          0.0003943  0.0029690 18.8190000   0.133  0.89575
## SpermCount   0.0013008  0.0004995 15.8880000   2.604  0.01925 *
## StatusS     -0.1415264  0.0932540 17.6740000  -1.518  0.14679
## StageB       0.2103049  0.0667399 10.3920000   3.151  0.00985 **
```

```
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn StatsS
## VAP         -0.962
## SpermCount -0.588  0.394
## StatusS     0.071 -0.101 -0.221
## StageB      0.071 -0.099 -0.180  0.191
```

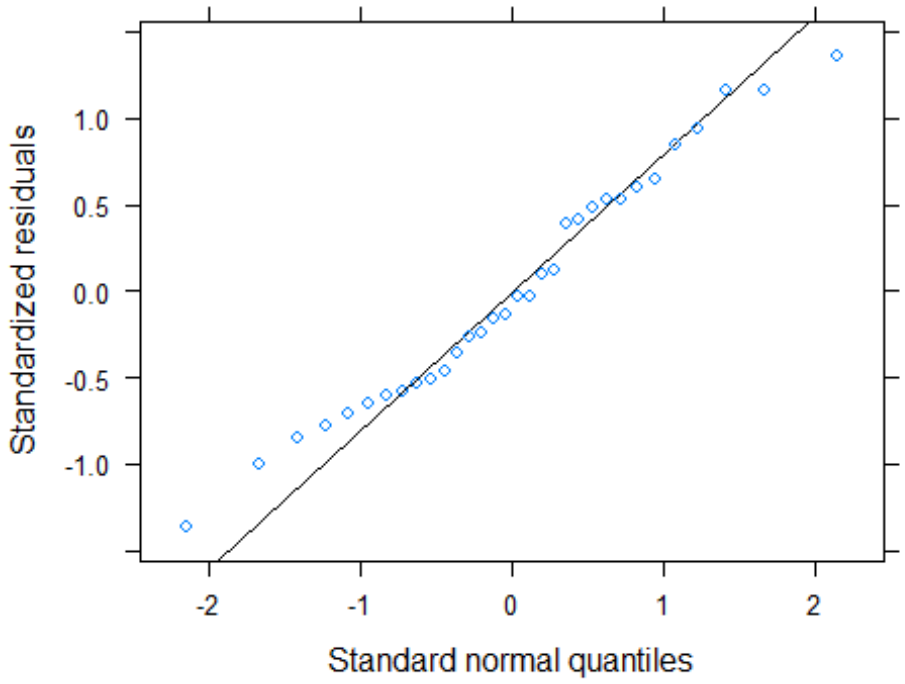
```
confint.merMod(modelP161,level=0.95,method="Wald")
```

```
##                      2.5 %       97.5 %
## .sig01                  NA           NA
## .sig02                  NA           NA
## .sigma                  NA           NA
## (Intercept) 18.9476932004 21.257373383
## VAP         -0.0054248501  0.006213533
## SpermCount   0.0003218507  0.002279810
## StatusS     -0.3243010079  0.041248111
## StageB       0.0794970300  0.341112744
```

```
plot(modelP161, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```




```
qqmath(modelP161)#Visual Check Normality assumption
```



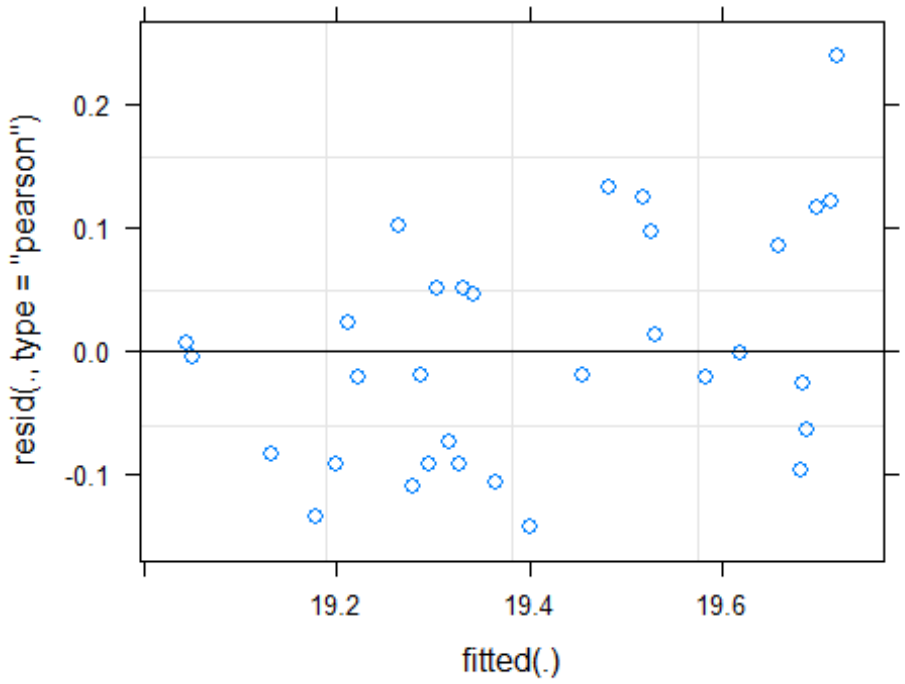

```
shapiro.test(resid(modelP161))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP161)
## W = 0.97409, p-value = 0.619

rand(modelP161)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##          Chi.sq Chi.DF p.value
## MaleID 3.09e+00      1    0.08 .
## Week   5.83e-06      1    1.00
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

proteins[[181]]

## [1] "B5XEM0_SALSA"

modelP181<-lmer(B5XEM0_SALSA ~ VAP + SpermCount + Status + Stage +
                  (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats
)
summary(modelP181)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
```

```
## Formula:
## B5XEM0_SALSA ~ VAP + SpermCount + Status + Stage + (1 | MaleID) +
##     (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 24.8
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.04591 -0.62728 -0.08906  0.43370  1.74818
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.046230 0.2150
##  Week     (Intercept) 0.006305 0.0794
##  Residual             0.018769 0.1370
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##              Estimate Std. Error        df t value Pr(>|t|)
## (Intercept) 18.741465   0.476677 22.318000  39.317   <2e-16 ***
## VAP          0.002324   0.002400 21.486000   0.968   0.3437
## SpermCount   0.001030   0.000389 16.983000   2.647   0.0170 *
## StatusS     -0.054906   0.072385 17.931000  -0.759   0.4580
## StageB      -0.107303   0.051126 12.195000  -2.099   0.0573 .
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn StatsS
## VAP         -0.962
## SpermCount -0.595  0.413
## StatusS     0.060 -0.088 -0.215
## StageB      0.070 -0.097 -0.179  0.195
```

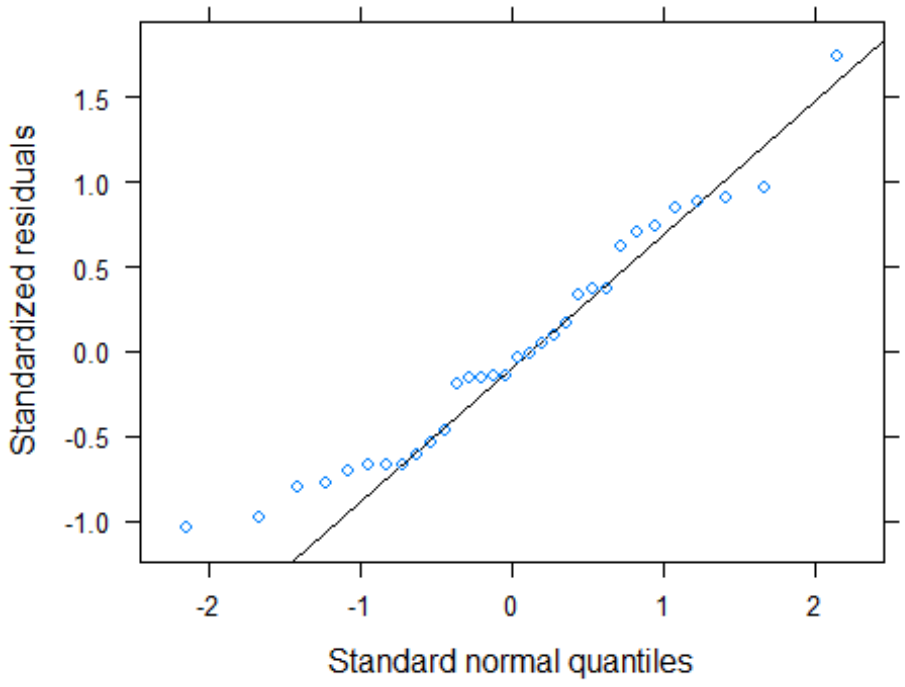
```
confint.merMod(modelP181,level=0.95,method="Wald")
```

```
##                      2.5 %       97.5 %
## .sig01                  NA           NA
## .sig02                  NA           NA
## .sigma                  NA           NA
## (Intercept) 17.8071952474 19.675733894
## VAP         -0.0023802936  0.007027356
## SpermCount   0.0002672139  0.001791990
## StatusS     -0.1967776408  0.086965160
## StageB      -0.2075089747 -0.007096788
```

```
plot(modelP181, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```




```
qqmath(modelP181)#Visual Check Normality assumption
```




```
shapiro.test(resid(modelP181))#Test Check Normality assumption
```

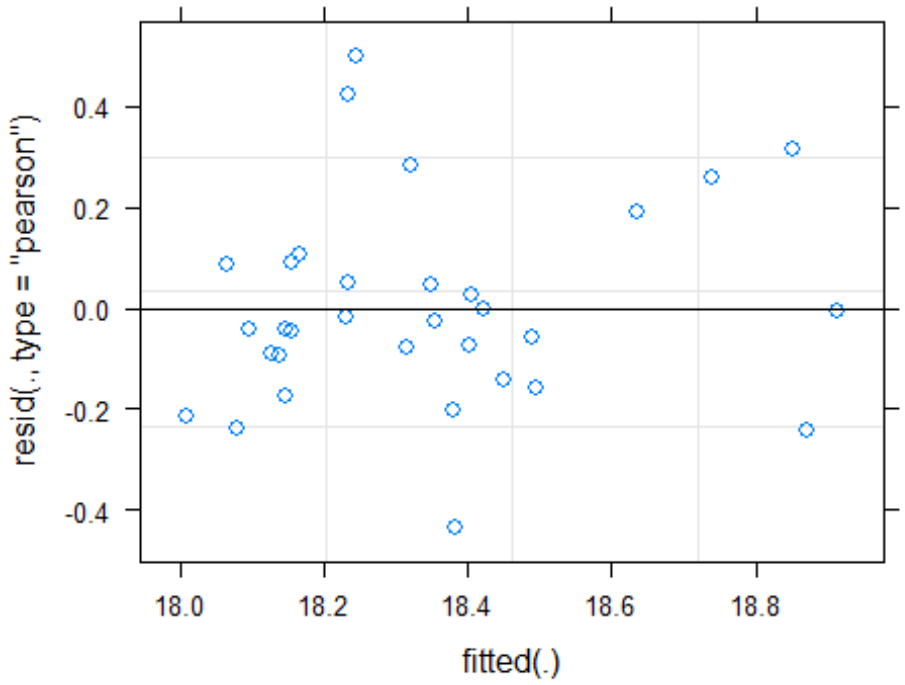
```
##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP181)
## W = 0.95582, p-value = 0.2104

rand(modelP181)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID  7.841      1   0.005 **
## Week    0.177      1   0.674
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

proteins[[182]]

## [1] "B5XEU8_SALSA"

modelP182<-lmer(B5XEU8_SALSA ~ VAP + SpermCount + Status + Stage +
                  (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats
)
summary(modelP182)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula:
## B5XEU8_SALSA ~ VAP + SpermCount + Status + Stage + (1 | MaleID) +
##     (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 34.9
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.8917 -0.4600 -0.1466  0.3808  2.1736
##
## Random effects:
##  Groups   Name        Variance  Std.Dev.
##  MaleID   (Intercept) 2.215e-18 1.488e-09
##  Week     (Intercept) 7.123e-02 2.669e-01
##  Residual             5.323e-02 2.307e-01
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##               Estimate Std. Error         df t value Pr(>|t|)
## (Intercept) 18.3527275  0.6029118 26.5820000  30.440   <2e-16 ***
## VAP         -0.0024695  0.0030664 25.5930000  -0.805   0.4280
## SpermCount   0.0013575  0.0005164 23.2350000   2.629   0.0149 *
## StatusS     -0.0276891  0.0862107 22.8090000  -0.321   0.7510
## StageB       0.0616618  0.0838397 22.7570000   0.735   0.4696
```

```
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn StatsS
## VAP         -0.944
## SpermCount -0.536  0.331
## StatusS      0.144 -0.169 -0.237
## StageB       0.060 -0.090 -0.199  0.119
```

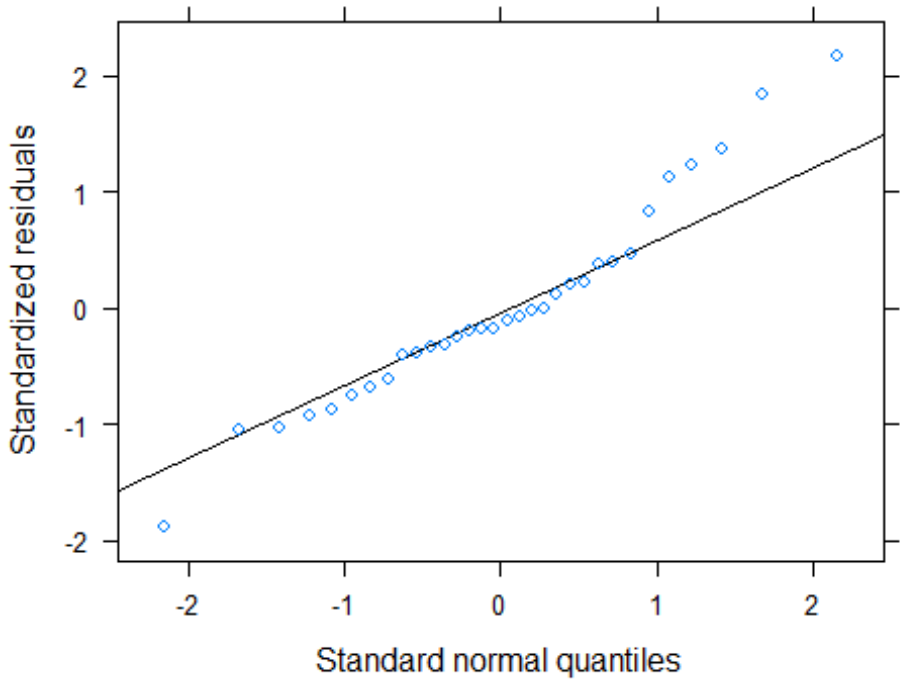
```
confint.merMod(modelP182,level=0.95,method="Wald")
```

```
##                      2.5 %       97.5 %
## .sig01                  NA           NA
## .sig02                  NA           NA
## .sigma                  NA           NA
## (Intercept) 17.1710420248 19.534412971
## VAP         -0.0084795729  0.003540512
## SpermCount   0.0003453347  0.002369636
## StatusS     -0.1966589338  0.141280717
## StageB      -0.1026608813  0.225984577
```

```
plot(modelP182, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```




```
qqmath(modelP182)#Visual Check Normality assumption
```



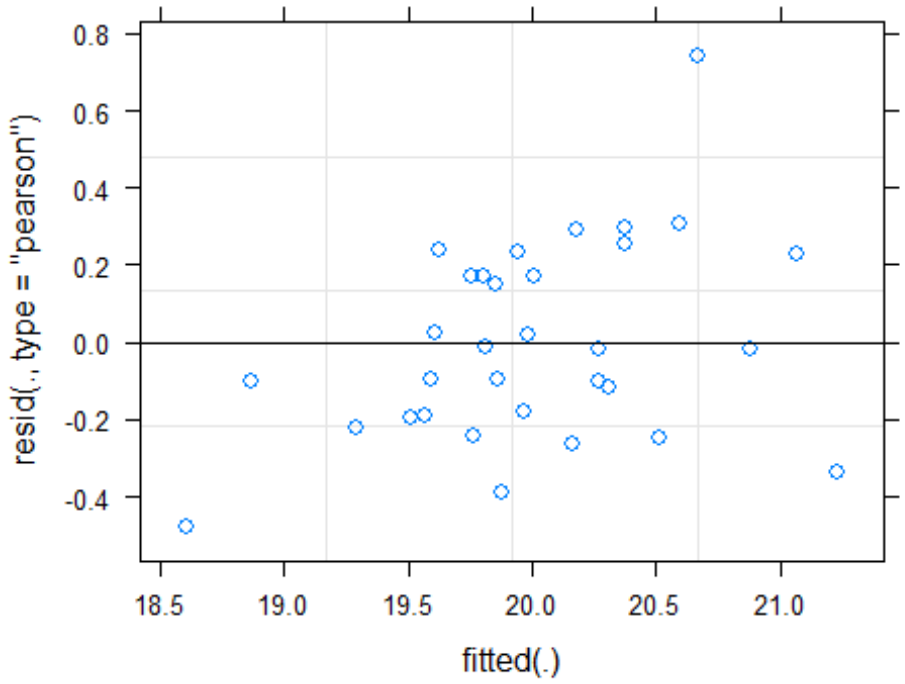

```
shapiro.test(resid(modelP182))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP182)
## W = 0.95856, p-value = 0.2508

rand(modelP182)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##          Chi.sq Chi.DF p.value
## MaleID 7.11e-14      1   1.000
## Week   7.27e+00      1   0.007 **
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

proteins[[198]]

## [1] "B8R4G1_ONCTS"

modelP198<-lmer(B8R4G1_ONCTS ~ VAP + SpermCount + Status + Stage +
                  (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats
)
summary(modelP198)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
```

```
## Formula:
## B8R4G1_ONCTS ~ VAP + SpermCount + Status + Stage + (1 | MaleID) +
##     (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 75.8
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.30573 -0.51940 -0.04235  0.50121  2.02158
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.2023   0.4497
##  Week     (Intercept) 0.2047   0.4524
##  Residual             0.1356   0.3683
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##              Estimate Std. Error        df t value Pr(>|t|)
## (Intercept) 17.719733   1.269009 23.901000  13.963 5.46e-13 ***
## VAP          0.010827   0.006388 22.598000   1.695   0.1039
## SpermCount   0.002359   0.001016 17.740000   2.321   0.0324 *
## StatusS     -0.454992   0.185393 19.418000  -2.454   0.0237 *
## StageB      -0.158904   0.136874 11.841000  -1.161   0.2685
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn StatsS
## VAP         -0.955
## SpermCount -0.584  0.408
## StatusS     0.072 -0.099 -0.223
## StageB      0.071 -0.097 -0.184  0.182
```

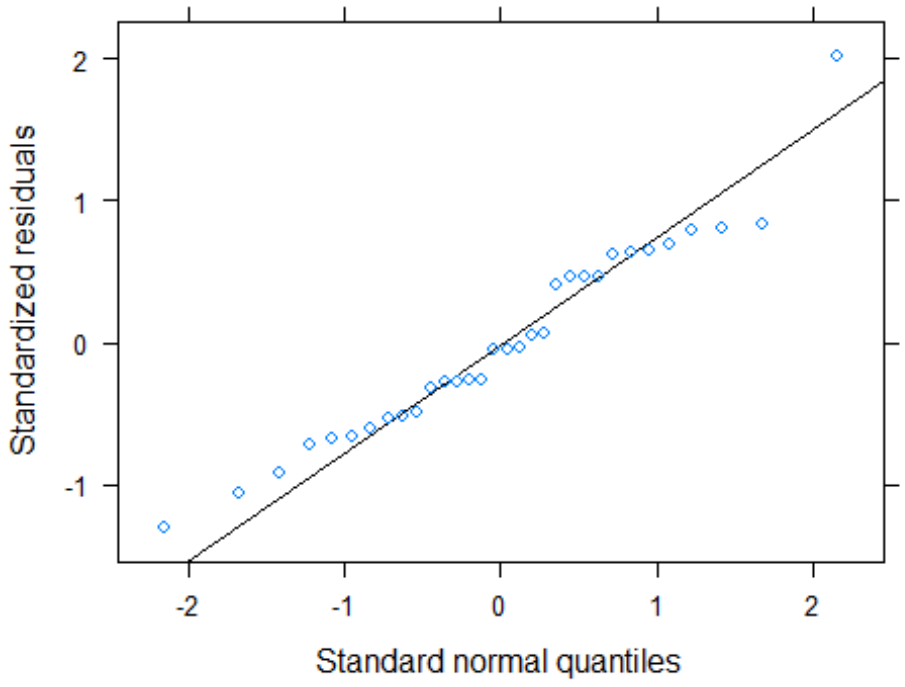
```
confint.merMod(modelP198,level=0.95,method="Wald")
```

```
##                      2.5 %       97.5 %
## .sig01                  NA           NA
## .sig02                  NA           NA
## .sigma                  NA           NA
## (Intercept) 15.2325215522 20.206944985
## VAP         -0.0016941463  0.023348276
## SpermCount   0.0003672689  0.004349943
## StatusS     -0.8183558480 -0.091628466
## StageB      -0.4271715057  0.109363756
```

```
plot(modelP198, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```




```
qqmath(modelP198)#Visual Check Normality assumption
```




```
shapiro.test(resid(modelP198))#Test Check Normality assumption
```

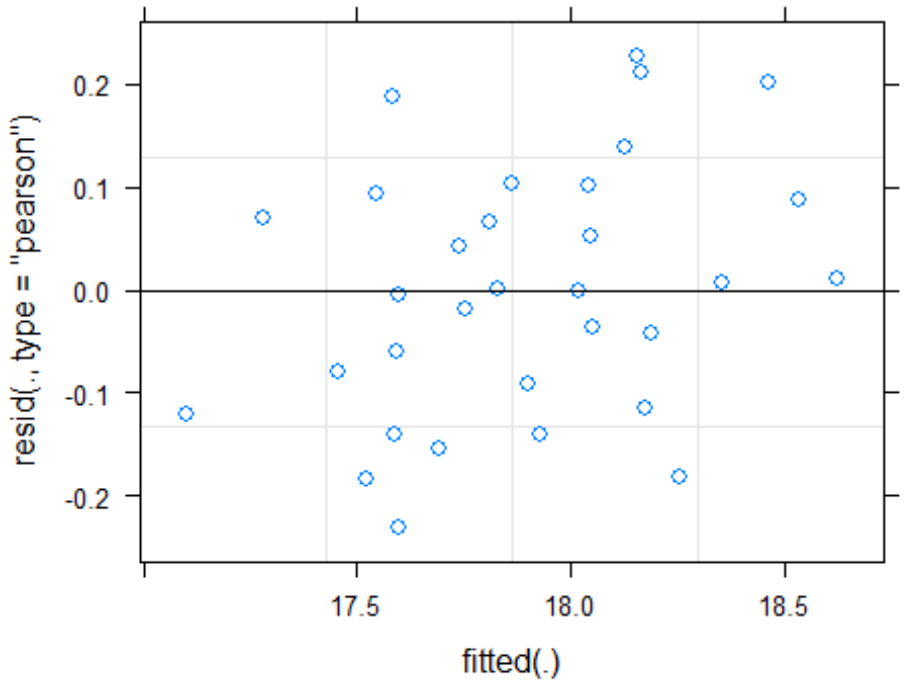
```
##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP198)
## W = 0.95892, p-value = 0.2566

rand(modelP198)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID   4.09      1    0.04 *
## Week     1.49      1    0.22
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

proteins[[206]]

## [1] "B9ELP5_SALSA"

modelP206<-lmer(B9ELP5_SALSA ~ VAP + SpermCount + Status + Stage +
                  (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats
)
summary(modelP206)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula:
## B9ELP5_SALSA ~ VAP + SpermCount + Status + Stage + (1 | MaleID) +
##     (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 43.7
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.25321 -0.52796 -0.00307  0.48412  1.23297
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.09359  0.3059
##  Week     (Intercept) 0.03202  0.1789
##  Residual             0.03423  0.1850
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##             Estimate Std. Error        df t value Pr(>|t|)
## (Intercept) 16.364649   0.670806 21.583000  24.396   <2e-16 ***
## VAP          0.005743   0.003368 20.620000   1.705   0.1032
## SpermCount   0.001222   0.000532 16.684000   2.297   0.0349 *
## StatusS      0.088409   0.098717 17.582000   0.896   0.3826
## StageB       0.189961   0.069126 12.355000   2.748   0.0173 *
```

```
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn StatsS
## VAP         -0.959
## SpermCount -0.598  0.427
## StatusS     0.052 -0.080 -0.210
## StageB      0.069 -0.096 -0.178  0.197
```

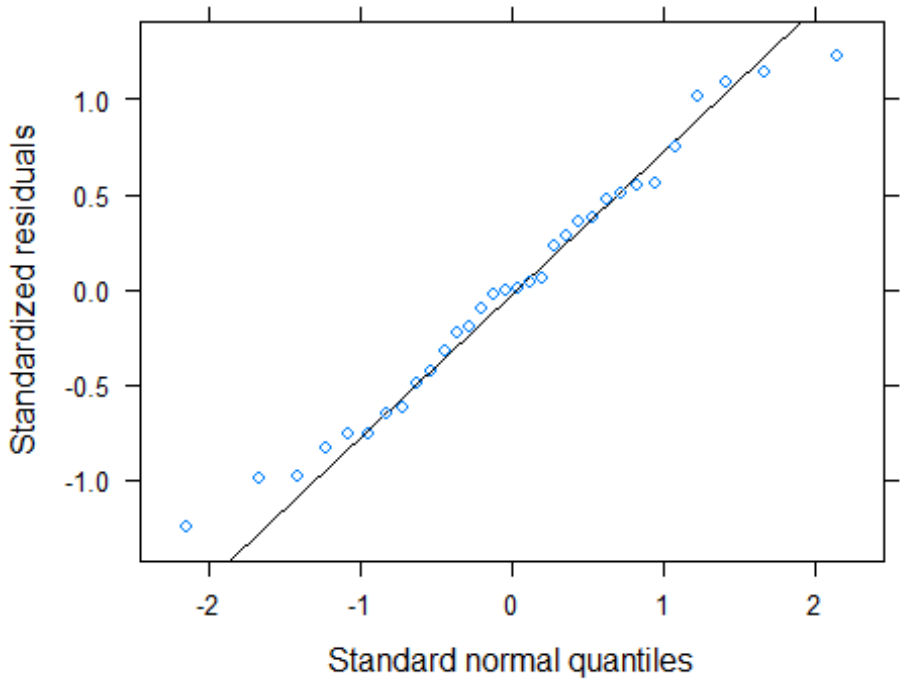
```
confint.merMod(modelP206,level=0.95,method="Wald")
```

```
##                      2.5 %       97.5 %
## .sig01                  NA           NA
## .sig02                  NA           NA
## .sigma                  NA           NA
## (Intercept) 15.0498943728 17.679403796
## VAP         -0.0008578049  0.012343471
## SpermCount   0.0001791842  0.002264718
## StatusS     -0.1050733193  0.281891817
## StageB       0.0544769269  0.325444203
```

```
plot(modelP206, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```




```
qqmath(modelP206)#Visual Check Normality assumption
```



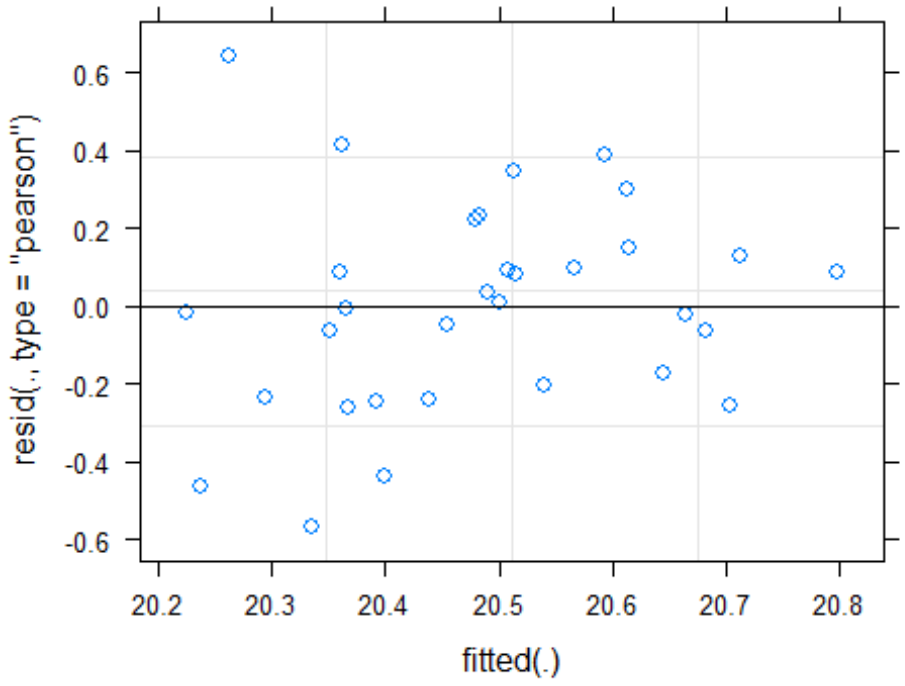

```
shapiro.test(resid(modelP206))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP206)
## W = 0.97555, p-value = 0.6639

rand(modelP206)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID  8.281      1   0.004 **
## Week    0.554      1   0.457
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

proteins[[243]]

## [1] "C0H9G4_SALSA"

modelP243<-lmer(C0H9G4_SALSA ~ VAP + SpermCount + Status + Stage +
                  (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats
)
summary(modelP243)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
```

```
## Formula:
## C0H9G4_SALSA ~ VAP + SpermCount + Status + Stage + (1 | MaleID) +
##     (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 43.8
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.88072 -0.69624  0.01116  0.44354  2.12849
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.007642 0.08742
##  Week     (Intercept) 0.003265 0.05714
##  Residual             0.091614 0.30268
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##               Estimate Std. Error         df t value Pr(>|t|)
## (Intercept) 19.4979662  0.6812427 18.4420000  28.621   <2e-16 ***
## VAP          0.0032387  0.0034697 17.0990000   0.933   0.3636
## SpermCount   0.0016694  0.0006788 24.9130000   2.459   0.0212 *
## StatusS     -0.1147023  0.1175018 22.9570000  -0.976   0.3392
## StageB      -0.1354902  0.1100000 12.0860000  -1.232   0.2415
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn StatsS
## VAP         -0.949
## SpermCount -0.543  0.289
## StatusS     0.150 -0.180 -0.238
## StageB      0.057 -0.091 -0.192  0.125
```

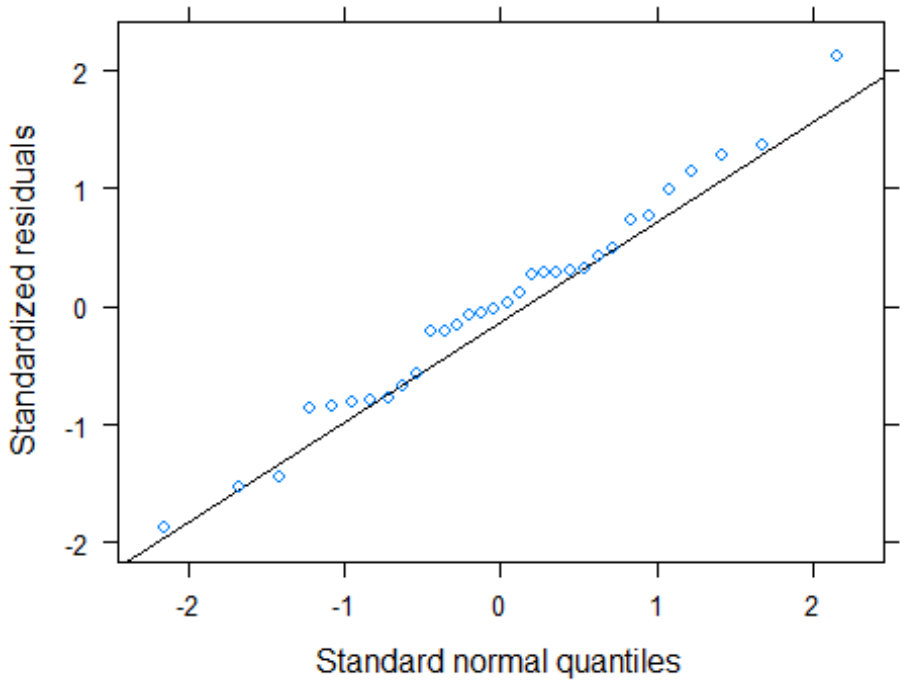
```
confint.merMod(modelP243,level=0.95,method="Wald")
```

```
##                      2.5 %       97.5 %
## .sig01                  NA           NA
## .sig02                  NA           NA
## .sigma                  NA           NA
## (Intercept) 18.1627549865 20.833177334
## VAP         -0.0035617849  0.010039128
## SpermCount   0.0003389057  0.002999842
## StatusS     -0.3450016622  0.115597055
## StageB      -0.3510862874  0.080105834
```

```
plot(modelP243, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```




```
qqmath(modelP243)#Visual Check Normality assumption
```




```
shapiro.test(resid(modelP243))#Test Check Normality assumption
```

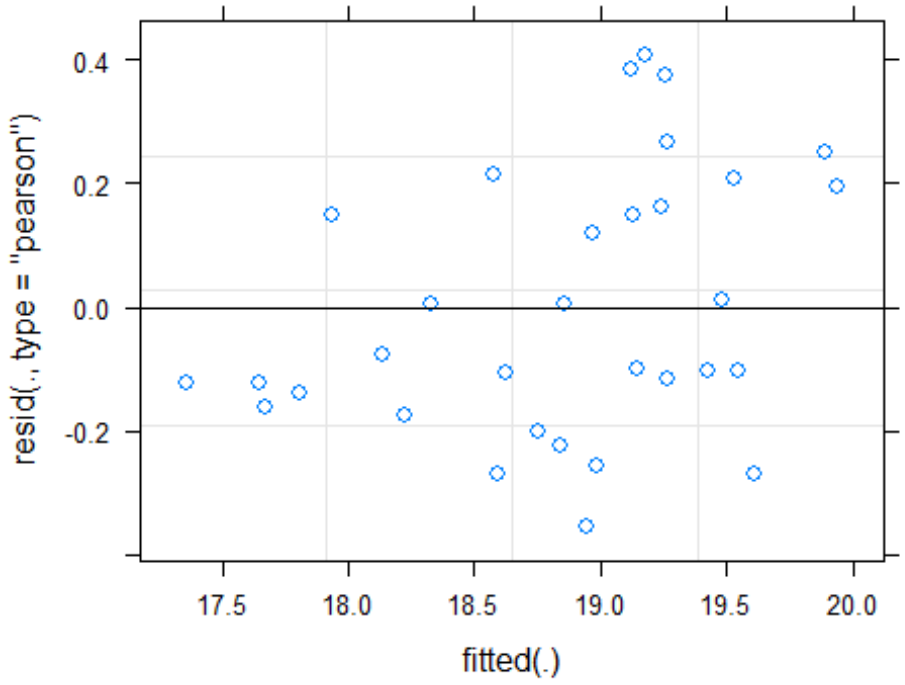
```
##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP243)
## W = 0.98564, p-value = 0.9367

rand(modelP243)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID 0.0522      1     0.8
## Week   0.0488      1     0.8

proteins[[298]]

## [1] "D2KVP8_ONCNE"

modelP298<-lmer(D2KVP8_ONCNE ~ VAP + SpermCount + Status + Stage +
                  (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats
)
summary(modelP298)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula:
## D2KVP8_ONCNE ~ VAP + SpermCount + Status + Stage + (1 | MaleID) +
##     (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 80.3
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.0869 -0.4440 -0.2654  0.5186  1.2474
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.5437   0.7373
##  Week     (Intercept) 0.0000   0.0000
##  Residual             0.1077   0.3282
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##               Estimate Std. Error         df t value Pr(>|t|)
## (Intercept) 20.2657421  1.2309439 19.7640000  16.464 5.28e-13 ***
## VAP         -0.0034512  0.0061547 18.8660000  -0.561  0.58158
## SpermCount  -0.0031392  0.0009728 14.3510000  -3.227  0.00592 **
## StatusS      0.3265390  0.1829601 14.5420000   1.785  0.09517 .
## StageB       0.1485562  0.1231373 11.2980000   1.206  0.25231
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn StatsS
## VAP         -0.963
## SpermCount -0.610  0.440
## StatusS     0.034 -0.065 -0.199
## StageB      0.068 -0.095 -0.172  0.209
```

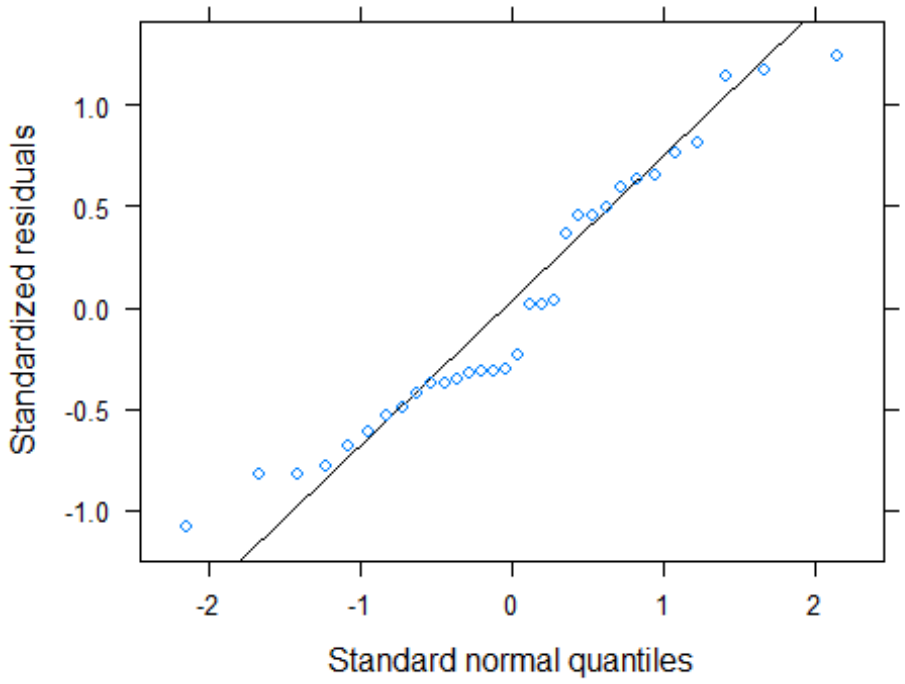
```
confint.merMod(modelP298,level=0.95,method="Wald")
```

```
##                     2.5 %       97.5 %
## .sig01                 NA           NA
## .sig02                 NA           NA
## .sigma                 NA           NA
## (Intercept) 17.853136466 22.678347777
## VAP         -0.015514231  0.008611906
## SpermCount  -0.005045786 -0.001232641
## StatusS     -0.032056258  0.685134245
## StageB      -0.092788422  0.389900832
```

```
plot(modelP298, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```




```
qqmath(modelP298)#Visual Check Normality assumption
```



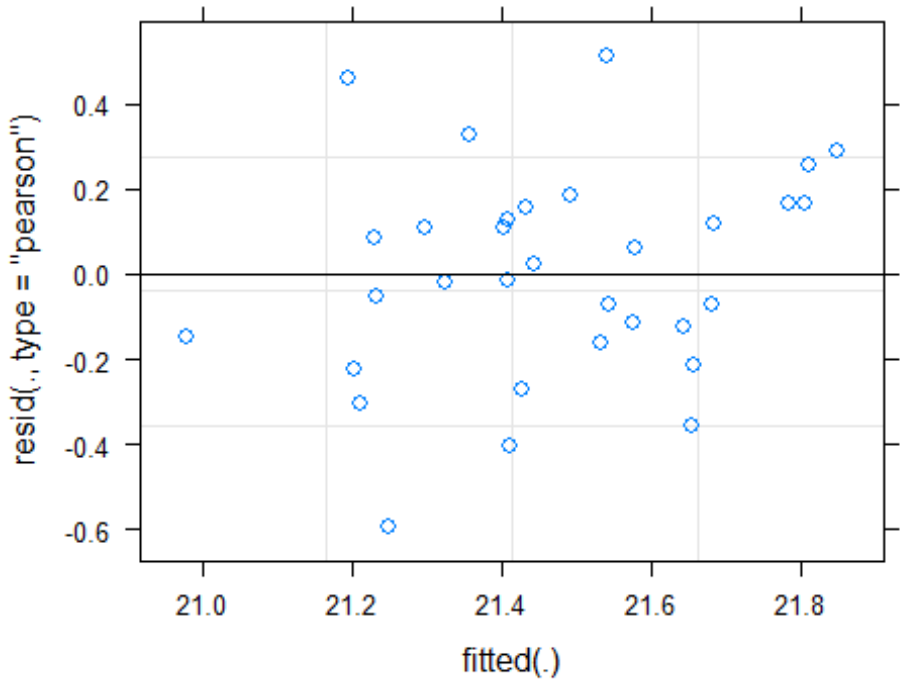

```
shapiro.test(resid(modelP298))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP298)
## W = 0.94242, p-value = 0.0877

rand(modelP298)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##          Chi.sq Chi.DF p.value
## MaleID 1.30e+01      1   3e-04 ***
## Week   9.95e-14      1       1
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

proteins[[305]]

## [1] "F8LFR3_ONCMY"

modelP305<-lmer(F8LFR3_ONCMY ~ VAP + SpermCount + Status + Stage +
                  (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats
)
summary(modelP305)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
```

```
## Formula:
## F8LFR3_ONCMY ~ VAP + SpermCount + Status + Stage + (1 | MaleID) +
##     (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 44.3
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -2.04972 -0.52184  0.02098  0.54108  1.76768
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.02041  0.1428
##  Week     (Intercept) 0.00000  0.0000
##  Residual             0.08450  0.2907
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##               Estimate Std. Error         df t value Pr(>|t|)
## (Intercept) 20.8692787  0.6835071 25.4580000  30.533   <2e-16 ***
## VAP          0.0019310  0.0034697 25.5400000   0.557   0.5827
## SpermCount   0.0015494  0.0006792 24.9860000   2.281   0.0313 *
## StatusS     -0.3197373  0.1201559 26.9990000  -2.661   0.0130 *
## StageB      -0.1979414  0.1060914 14.3040000  -1.866   0.0827 .
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn StatsS
## VAP         -0.950
## SpermCount -0.548  0.297
## StatusS     0.140 -0.171 -0.240
## StageB      0.062 -0.095 -0.191  0.137
```

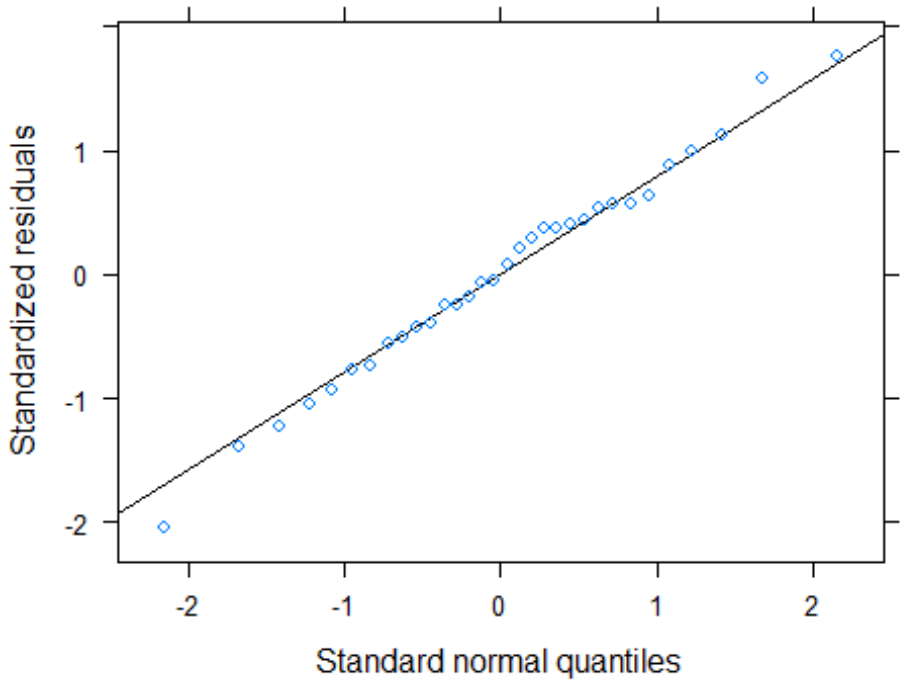
```
confint.merMod(modelP305,level=0.95,method="Wald")
```

```
##                      2.5 %       97.5 %
## .sig01                  NA           NA
## .sig02                  NA           NA
## .sigma                  NA           NA
## (Intercept) 19.5296293426 22.208928002
## VAP         -0.0048695981  0.008731534
## SpermCount   0.0002181471  0.002880636
## StatusS     -0.5552385389 -0.084236085
## StageB      -0.4058767138  0.009993997
```

```
plot(modelP305, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```




```
qqmath(modelP305)#Visual Check Normality assumption
```




```
shapiro.test(resid(modelP305))#Test Check Normality assumption
```

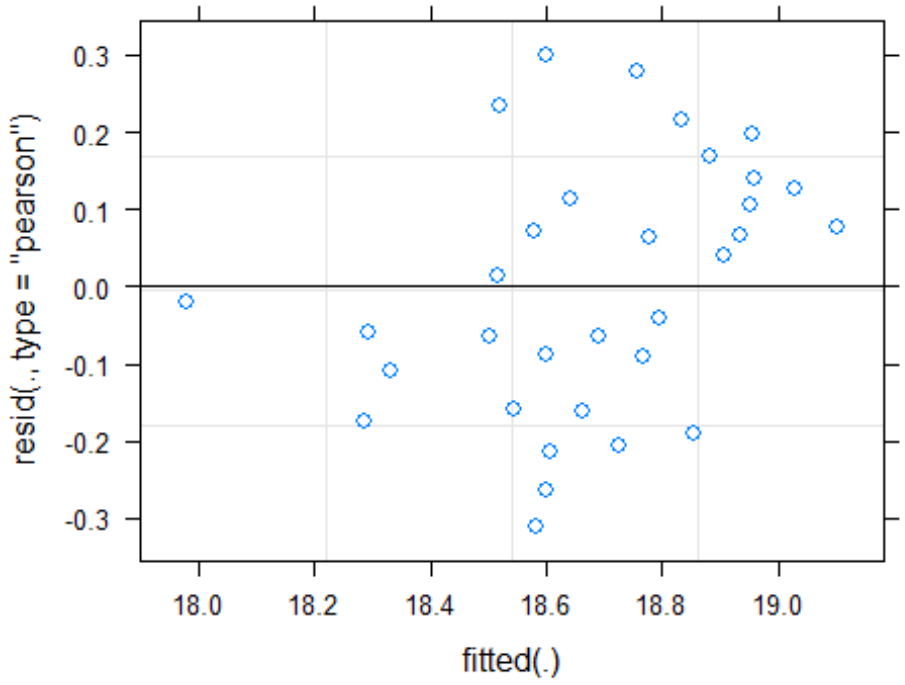
```
##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP305)
## W = 0.99141, p-value = 0.9953

rand(modelP305)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##          Chi.sq Chi.DF p.value
## MaleID 4.38e-01      1     0.5
## Week   9.24e-14      1     1.0

proteins[[315]]

## [1] "Q4QZ18_ONCMY"

modelP315<-lmer(Q4QZ18_ONCMY ~ VAP + SpermCount + Status + Stage +
                  (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats
)
summary(modelP315)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula:
## Q4QZ18_ONCMY ~ VAP + SpermCount + Status + Stage + (1 | MaleID) +
##     (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 40.7
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -1.43259 -0.55713 -0.01605  0.54117  1.38355
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.02962  0.1721
##  Week     (Intercept) 0.06869  0.2621
##  Residual             0.04736  0.2176
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##               Estimate Std. Error         df t value Pr(>|t|)
## (Intercept) 20.2738894  0.6794376 26.4220000  29.839  < 2e-16 ***
## VAP         -0.0069298  0.0034401 25.5090000  -2.014  0.05462 .
## SpermCount  -0.0016843  0.0005596 20.9070000  -3.010  0.00669 **
## StatusS      0.1458526  0.0993601 22.3040000   1.468  0.15609
## StageB       0.0814251  0.0802192 11.9220000   1.015  0.33025
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn StatsS
## VAP         -0.952
## SpermCount -0.564  0.376
## StatusS     0.102 -0.127 -0.235
## StageB      0.070 -0.097 -0.192  0.159
```

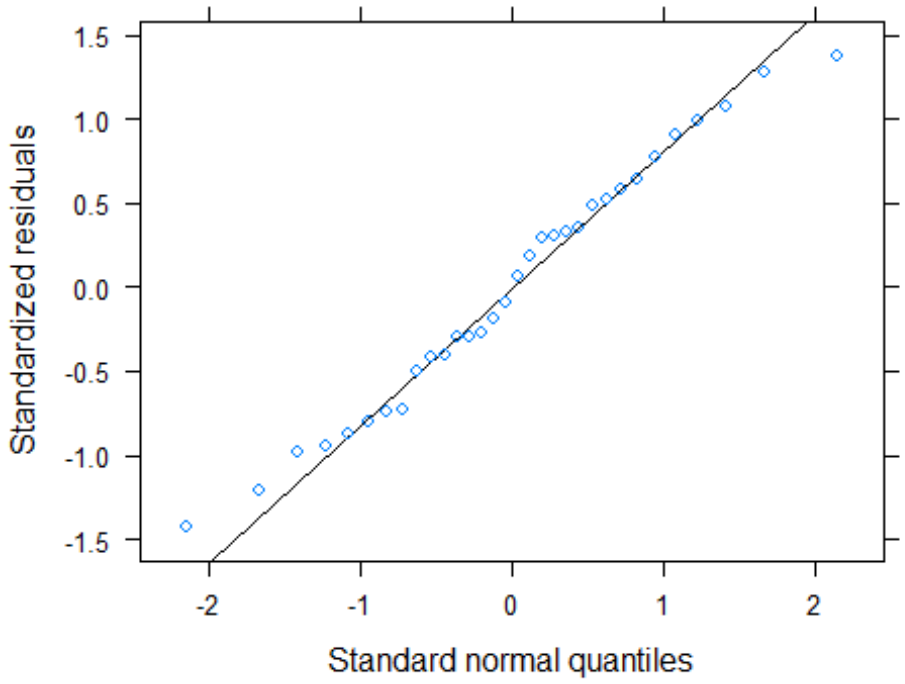
```
confint.merMod(modelP315,level=0.95,method="Wald")
```

```
##                     2.5 %        97.5 %
## .sig01                 NA            NA
## .sig02                 NA            NA
## .sigma                 NA            NA
## (Intercept) 18.942216076 21.6055626303
## VAP         -0.013672258 -0.0001873631
## SpermCount  -0.002781036 -0.0005875894
## StatusS     -0.048889696  0.3405948194
## StageB      -0.075801712  0.2386518584
```

```
plot(modelP315, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```




```
qqmath(modelP315)#Visual Check Normality assumption
```



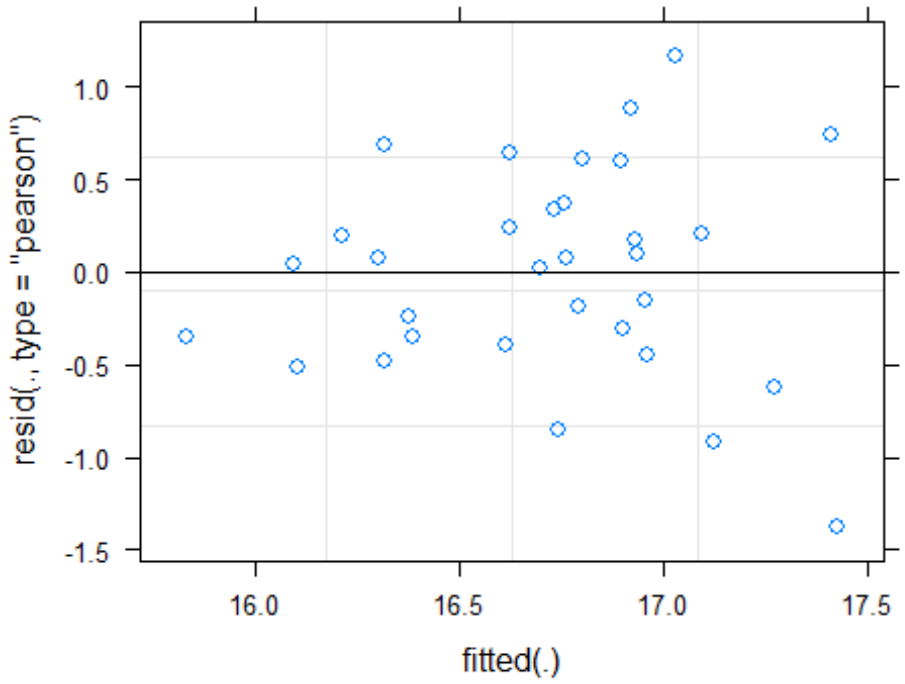

```
shapiro.test(resid(modelP315))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP315)
## W = 0.97739, p-value = 0.721

rand(modelP315)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID   1.12      1     0.3
## Week     1.78      1     0.2

proteins[[321]]

## [1] "Q6R4A2_ONCMY"

modelP321<-lmer(Q6R4A2_ONCMY ~ VAP + SpermCount + Status + Stage +
                  (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats
)
summary(modelP321)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula:
## Q6R4A2_ONCMY ~ VAP + SpermCount + Status + Stage + (1 | MaleID) +
```

```
##     (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 78.7
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -2.27596 -0.60091  0.09499  0.57099  1.93359
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  MaleID   (Intercept) 0.0000   0.0000
##  Week     (Intercept) 0.0000   0.0000
##  Residual             0.3675   0.6062
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##              Estimate Std. Error        df t value Pr(>|t|)
## (Intercept) 20.143145   1.252377 27.000000  16.084 2.22e-15 ***
## VAP         -0.013324   0.006357 27.000000  -2.096   0.0456 *
## SpermCount  -0.004177   0.001310 27.000000  -3.189   0.0036 **
## StatusS      0.373474   0.224838 27.000000   1.661   0.1083
## StageB       0.124550   0.219498 27.000000   0.567   0.5751
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn StatsS
## VAP         -0.944
## SpermCount -0.541  0.270
## StatusS     0.157 -0.190 -0.235
## StageB      0.052 -0.090 -0.191  0.117
```

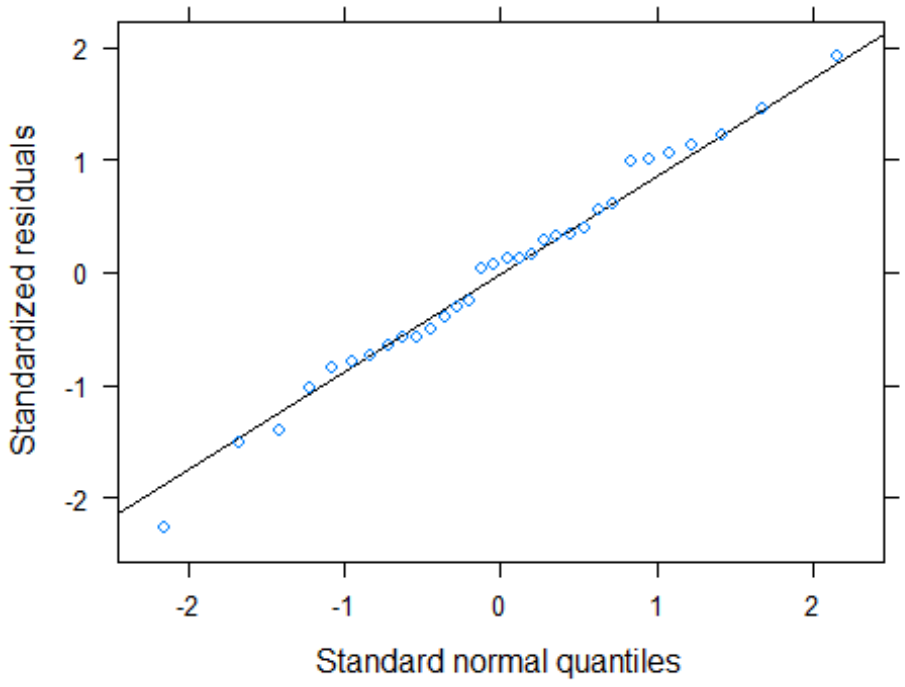
```
confint.merMod(modelP321,level=0.95,method="Wald")
```

```
##                     2.5 %        97.5 %
## .sig01                 NA            NA
## .sig02                 NA            NA
## .sigma                 NA            NA
## (Intercept) 17.688531000 22.5977580925
## VAP         -0.025784469 -0.0008640735
## SpermCount  -0.006743223 -0.0016098015
## StatusS     -0.067199923  0.8141483046
## StageB      -0.305658148  0.5547583045
```

```
plot(modelP321, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```




```
qqmath(modelP321)#Visual Check Normality assumption
```




```
shapiro.test(resid(modelP321))#Test Check Normality assumption
```

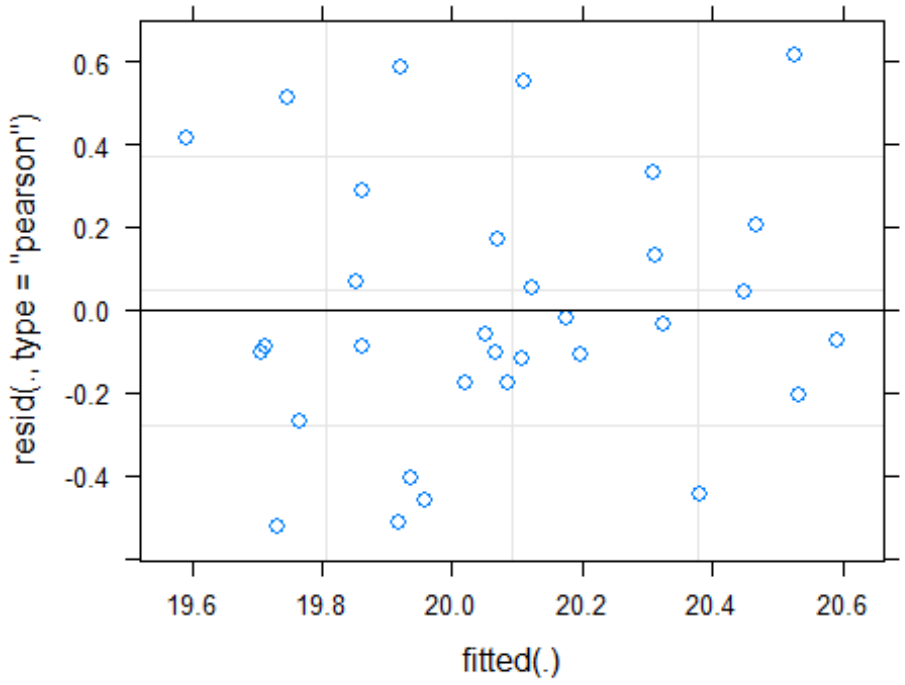
```
##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP321)
## W = 0.98957, p-value = 0.9858

rand(modelP321)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##        Chi.sq Chi.DF p.value
## MaleID      0      1       1
## Week        0      1       1

proteins[[348]]

## [1] "X5IE94_ONCKE"

modelP348<-lmer(X5IE94_ONCKE ~ VAP + SpermCount + Status + Stage +
                  (1|MaleID)+ (1|Week),data=RelativeabundanceafterNormMSstats
)
summary(modelP348)

## Linear mixed model fit by REML t-tests use Satterthwaite approximations
##   to degrees of freedom [lmerMod]
## Formula:
## X5IE94_ONCKE ~ VAP + SpermCount + Status + Stage + (1 | MaleID) +
##     (1 | Week)
##    Data: RelativeabundanceafterNormMSstats
##
## REML criterion at convergence: 51.7
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.4938 -0.4982 -0.1925  0.5071  1.7537
##
## Random effects:
##  Groups   Name        Variance  Std.Dev.
##  MaleID   (Intercept) 1.181e-16 1.087e-08
##  Week     (Intercept) 1.895e-02 1.377e-01
##  Residual             1.240e-01 3.521e-01
## Number of obs: 32, groups:  MaleID, 17; Week, 5
##
## Fixed effects:
##               Estimate Std. Error         df t value Pr(>|t|)
## (Intercept) 20.3136445  0.8135690 24.2940000  24.969  < 2e-16 ***
## VAP          0.0003749  0.0041687 23.6840000   0.090  0.92910
## SpermCount  -0.0017649  0.0007741 25.1830000  -2.280  0.03134 *
## StatusS      0.1736829  0.1310800 23.9420000   1.325  0.19767
## StageB       0.4250478  0.1277143 23.7500000   3.328  0.00284 **
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
##
## Correlation of Fixed Effects:
##             (Intr) VAP    SprmCn StatsS
## VAP         -0.952
## SpermCount -0.540  0.299
## StatusS     0.153 -0.181 -0.237
## StageB      0.055 -0.088 -0.195  0.118
```

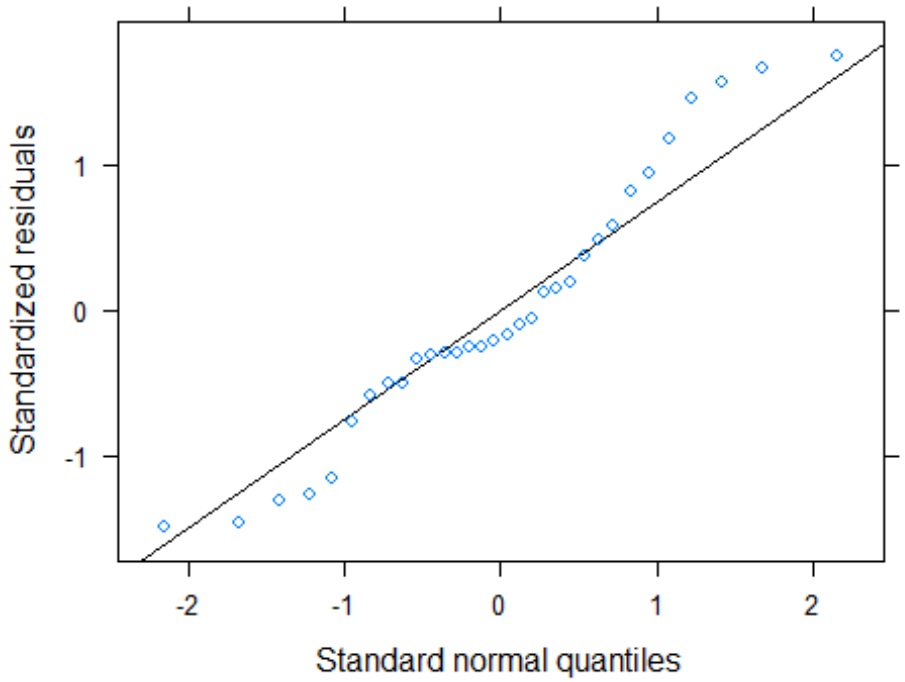
```
confint.merMod(modelP348,level=0.95,method="Wald")
```

```
##                     2.5 %        97.5 %
## .sig01                 NA            NA
## .sig02                 NA            NA
## .sigma                 NA            NA
## (Intercept) 18.719078586 21.9082104542
## VAP         -0.007795652  0.0085454496
## SpermCount  -0.003282065 -0.0002477379
## StatusS     -0.083229233  0.4305950911
## StageB       0.174732426  0.6753631132
```

```
plot(modelP348, results="hide", fig.show='hide')#Visual Check Variance assump
tion
```




```
qqmath(modelP348)#Visual Check Normality assumption
```




```
shapiro.test(resid(modelP348))#Test Check Normality assumption

##
##  Shapiro-Wilk normality test
##
## data:  resid(modelP348)
## W = 0.95312, p-value = 0.1766

rand(modelP348)#Test for significance of random predictor male ID

## Analysis of Random effects Table:
##          Chi.sq Chi.DF p.value
## MaleID 8.53e-14      1     1.0
## Week   9.15e-01      1     0.3
```